Pain during topical photodynamic therapy

by

Patrycja Mikolajewska

Institute for Cancer Research
Department of Radiation Biology, Oslo University Hospital, Radium Hospital and
University of Oslo

Series of dissertations submitted to the
Faculty of Mathematics and Natural Sciences, University of Oslo
No. 1105

ISSN 1501-7710

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

Produced in co-operation with Unipub.
The thesis is produced by Unipub merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

List of contents

Abbreviations

AIS2Pc	Aluminium disulfonated phthalocyanine
AK	Actinic keratosis
ALA	5-aminolevulinc acid
BCC	Basal cell carcinoma
BD	Bowen's disease
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
FCS	Foetal calf serum
FST I-V	Fitzpatrick skin type I-V
GABA	Gamma aminobutyric acid
Hp	Hematoporphyrin
HpD	Hematoporphyrin derivative
IL4	Interleukin 4
IL10	Interleukin 10
i.p.	Intra peritoneal
LED	Light emitting diode
MAL	Methyl 5-aminolevulinate
NO	Nitric oxide
PBS	Phosphate buffer saline
PDT	Photodynamic therapy
PpIX	Protoporphyrin IX
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SCC	Squamous cell carcinoma
SF	L(R)-sulforaphane
SIA	Subcutaneous infiltration anesthesia
TRPM8	Transient receptor potential cation channel, subfamily M, member 8
TRPV1	Transient receptor potential cation channel, subfamily V, member1
VAS	Visual analog scale

List of publications

1. The effect of lidocaine on PpIX photobleaching and outcome of ALA-PDT in vitro. **P. Mikolajewska**, A. Juzeniene, J. Moan
Photodiagnosis and Photodynamic Therapy 2007; 4(4):249-253.

2. Effect of (R)L-sulforaphane on 5-aminolevulinic acid-mediated photodynamic therapy. **P. Mikolajewska**, A. Juzeniene, J. Moan
Translational Research 2008; 152 (3):128-33.

3. Topical aminolaevulinic acid- and aminolaevulinic acid methyl ester-based photodynamic therapy with red and violet light: influence of wavelength on pain and erythema. **P.Mikolajewska**, V.Iani, A.Juzeniene, J.Moan
British Journal of Dermatology 2009; 161:1173–1179

4. Microneedle pre-treatment of human skin improves 5-aminolevulininc acid (ALA) and 5-aminolevulinic acid methyl ester (MAL) induced PpIX production for topical photodynamic therapy without increase in pain or erythema.
P. Mikolajewska, R.F. Donnelly, M. J. Garland, D. I.J. Morrow, T.R.R. Singh, V. Iani, J.Moan, A. Juzeniene,
Pharmaceutical Research 2010; 27(10): 2213-20

5. Bioimpedance for pain monitoring during cutaneous photodynamic therapy. Preliminary study. **P. Mikolajewska**, O. T. Rømoen, Ø. G. Martinsen, V. Iani, J. Moan, S. Grimnes, A. Juzeniene,
Photodiagnosis and Photodynamic Therapy 2011; doi: 10.1016/j.pdpdt.2011.06.001

1. Aims

Topical photodynamic therapy (PDT) has become an established treatment modality for dermatologic conditions like actinic keratosis, Bowen's disease, *in situ* squamous cell carcinoma (SCC) and superficial basal cell carcinoma (BCC). Pain during light exposure is one of the main disadvantages of PDT. This thesis undertakes different approaches to monitoring and managing the pain problem.

1. The influence of topical anaesthetic lidocaine, which is used in clinical PDT practice, and which may also act as a singlet oxygen quencher, was investigated on protoporphyrin IX (PpIX) photobleaching rate and the PDT effectiveness *in vitro* (paper I).

2. The influence of topical anti-erythemal substance L-sulforaphane on PpIX production and pain induction during PDT was examined in vitro and in vivo (paper II).

3. Different light wavelengths (violet light at 407 nm versus red light at 636 nm) for activation of PpIX were compared with regard to pain induction (paper III).

4. The use of a penetration enhancing device, microneedles, was evaluated with respect to the side effects of PDT (paper IV).

5. Bioimpedance spectroscopy was employed to investigate changes related to pain induction in the skin during light exposure in topical PDT (paper V).

6. A novel method for pain monitoring, namely, measuring time from the beginning of light exposure until the onset of pain, was evaluated in terms of its reproducibility and reliability (papers III-V).

2. Introduction

2.1 Topical photodynamic therapy (PDT)

The story of photodynamic therapy (PDT) started around the turn of the last century, when Oscar Raab, Medical student of Hermann von Tappeiner, noticed that acridine in combination with sunlight was lethal to *paramecium caudatum (1-3).* This finding inspired von Tappeiner to continue research on light and dye induced cell death. Soon after, in cooperation with Albert Jesionek, a young assistant professor at the Department of Dermatology, University of Munich, von Tappeiner discovered that the presence of oxygen in the system was necessary for photosensitization to occur. They were the first to see the antitumor potential of photosensitization and the first to treat humans by applying eosin and exposing to white light lupus vulgaris, stage II syphilis and basal cell carcinoma (BCC) (4;5). It was about that time von Tappeiner, together with Albert Jodlbauer, introduced the term "photodynamic" to distinguish the process from already well known photo-processes occurring in photography (6;7).In 1911, Hausmann injected hematoporphyrin (Hp) into mice and observed erythema and oedema after sunlight exposure (8). Hp was recognized as a powerful photosensitizer and studied in detail. Its components were described, tumor localizing properties were determined and, finally, its derivatives were produced (9-12). The research took off and, in the 1970s, a big number of scientists joined the quest for a perfect photosensitizer. The milestones in PDT development are shown in Table 1.

Table 1 Milestones in the development of PDT (1900-2010).

Year	Scientist/Company	Discovery
1900	O. Raab (2)	Combination of light and acridine was toxic to paramecium.
1902	Ledoux-Lebards (13)	Eosin kills paramecia better in oxygen enriched flasks.
1903	von Tappeiner and Jesionek (7)	Demonstrated requirement of oxygen in photosensitization.
1911	W.Hausmann (8)	Hematoporphyrin a powerfull photosensitizer
1912	Meyer-Betz (14)	First injection of hematoporphyrin (into his own veins)
1948-1968	Shenek and Foote (15)	The role of singlet oxygen was recognized. Photosensitization was divided in two mechanisms, Type I and type II reactions.
1950s	Figge *et al.* (16)	Hp has tumor localizing properties
1951	Berlin *et al.* (17)	Excess administration of exogenous ALA bypasses the cellular feedback control mechanism and leads to PpIX accumulation.
1955	Schwartz (18)	Hp needs purification; Hp derivative with sulfuric acid and acetic acids

1961	R.L. Lipson (19)	HpD has more components than Hp, but better tumor localizing properties than Hp
1970s	A large number of scientists joined in the search for the best photosensitizer	
1979	Moore (20)	Biochemistry of porphyria showed that porphyrins can be produced endogenously.
1985-1992	Kessel *et al.* (21)	Diethers and diesters were central. Aggregates show tumor localizing properties
1987	Malik and Lugaci (22)	Use of ALA in PDT
1987	Peng Q. (23)	ALA has tumor localizing properties.
1987	Mang *et al.* (24)	Photobleaching of porphyrins used in PDT
1990	Kennedy (25)	First clinical trial with ALA for skin abnormalities.
1990-1991	Moan *et al. (26)*	Short lifetime of singlet oxygen explains why PDT occurs close to cell and why PDT has low genotoxic potential.
1998	Dougherty (27)	Purifies Hp chromatographically to Photofrin. Still most widely used in PDT. However Photofrin has drawbacks: contamination with impurities, low absorbance at 630 nm, prolonged skin photosensitivity (6-8 weeks)
1999	DUSA Pharmaceuticals (28)	ALA under the name Levulan is approved for the treatment of actinic keratosis (AK).
2002	PhotoCure ASA (29)	Metvix is a photosensitive cream containing the methyl ester of 5-aminolevulinic acid, which has been developed and launched by PhotoCure in Sweden for the treatment of high-risk basal cell carcinoma and actinic keratosis
2008	Photocure ASA, Galderma (30)	Metvixia (Metvix outside US and France) in combination with Aktilite CL128, the LED based narrow band (630 nm) red light technology device has been approved by US Food and Drug Administration (FDA) for the treatment of actinic keratosis (AK), a pre-cancerous skin lesion.

As of today, it is known that PDT requires a target-site-localizing photosensitizer, light at a specific wavelength and the presence of oxygen for effectiveness. Topical PDT is widely studied and approved for non-melanoma conditions (31;32).

2.2 ALA and MAL

2.2.1 ALA

Ever since 5-aminolevulinic acid (ALA) was first used for topical photodynamic application, it has been an exciting therapeutic modality for dermatologists. Due to its small molecular weight (167.6 Da), the ALA molecules can penetrate through *stratum corneum* (33;34). Moreover, ALA is a natural compound with a fast clearance from the body (about

24- 48 h) leading to low systemic toxicity (27). Although ALA is a hydrophilic molecule, since early 1990s, many clinical studies have been published for actinic keratosis, Bowen's disease, superficial BCC and SCC, demonstrating a clearance rate of 80-100%, 90-100%, 80-100% and 67-92%, respectively (35).

As ALA passes through *stratum corneum* and enters into neoplastic cells, it interacts with the heme biosynthesis pathway which takes place in every cell of the body (with the exception of erytrocytes) and is very tightly regulated. Normally, the concentration of intermediate products is too low for photosensitization. When exogenous ALA enters into a cell, it bypasses the negative feedback controls allowing for the photosensitizer to accumulate (figure 1).

The heme synthesis pathway has two rate-limiting steps. The first one is the condensation of succynyl coenzyme A (succynyl CoA) and glicyne into ALA. Addition of exogenous ALA bypasses this process. The second rate-limiting step is the incorporation of iron into the PpIX chain. This step is catalyzed by the enzyme ferrochelataze. However, since there is an overabundance of ALA and, thus, PpIX and only a limited amount of iron, the PpIX accumulates in concentrations sufficient for photosensitization.

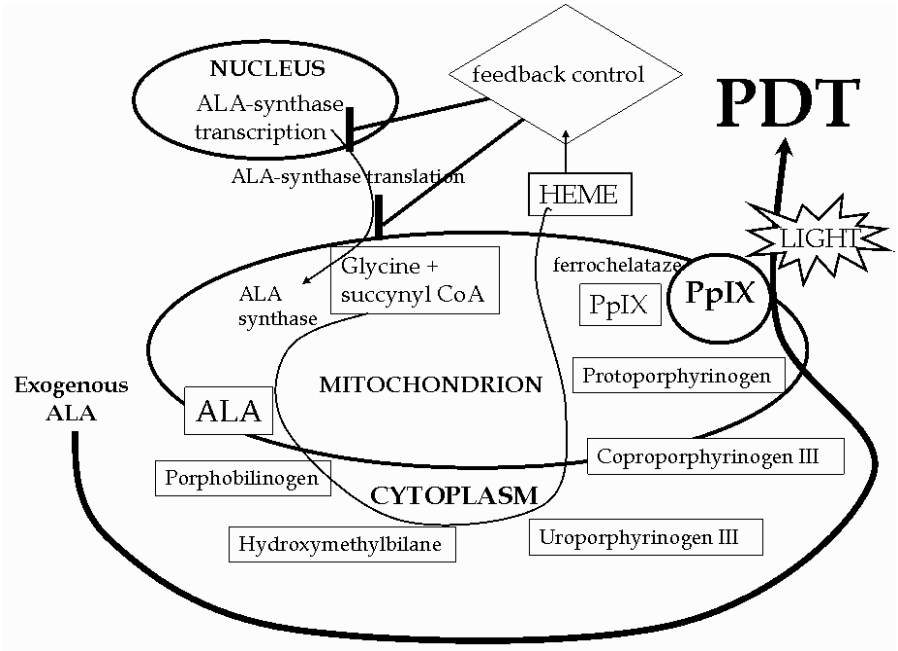


Figure 1. Heme biosynthesis pathway with marked pathway of PpIX accumulation after addition of exogenous ALA. Adapted from Kufe *et al.* (36)

In addition to being natural, chemically pure and having minimal dark toxicity, PpIX has another advantage: a part of its absorption spectrum lies in the tissue optical window (600- 1300 nm). Thus, activation with deeper penetrating light wavelengths is possible (figure 2). When excited by the absorbed light, PpIX can decay back to its ground state either by emitting fluorescence (this phenomenon is widely used for tumor visualization and diagnosis) or by reacting with the surrounding biomolecules or molecular oxygen. The latter reactions lead to formation of reactive radicals, mainly singlet oxygen, which, in turn, react with lipids, proteins, nucleic acid etc. causing lethal damages to cells.

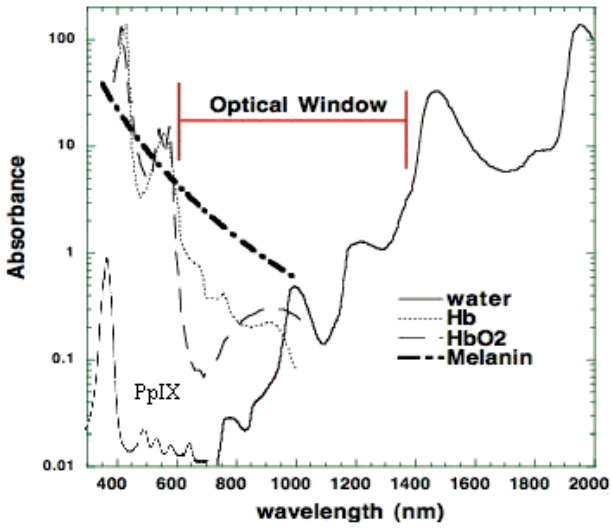


Figure 2. Tissue optical window (i.e. range of wavelengths where light has maximum depth of penetration into tissue) and the protoporphyrin IX (PpIX), hemoglobin (Hb), oxyhemoglobin (HbO2), melanin and water absorbance spectra. Based on Hamblin,M.R. (37)

2.2.2 MAL

The two main drawbacks of ALA-PDT are the shallow penetration depth of ALA and transient local symptoms during and after light exposure, like stinging, burning, itching, erythema and edema. To counteract these limitations the methyl ester of ALA (MAL) was introduced.

MAL, being more lipophilic than ALA, was thought to penetrate deeper into tissues (23;38). However, it has been shown that MAL-induced PpIX is being produced with a time lag (39). Possibly, MAL is bound to *stratum corneum* and other lipophilic compartments, which significantly slows down its penetration (40). Therefore higher concentrations of

MAL are needed for equal production of PpIX as from ALA in normal healthy skin (41). On the other hand, MAL has better tumor localizing properties and does not lead to systemic photosensitization after local topical application (39). It has also been shown that MAL causes less pain sensations than ALA (42). Although not all findings support this statement (43), it is generally believed that MAL-PDT is less painful than ALA-PDT due to its transport mechanisms (44) and tissue localization pattern (45;46). ALA has a structure that is similar to aminobutyric acid (GABA), and might be transported into a cell by the same carrier systems as this neurotransmitter (44). MAL, on the other hand, might be taken up by more than one active transport mechanism (47). The larger uptake of ALA into nerve cells via GABA receptors may explain the more intense pain experienced by patients during ALA-PDT as compared to MAL-PDT (44).

MAL, under the name Metvix, is approved in Americas for the treatment of actinic keratosis and many countries in Europe and Asia for the treatment of actinic keratosis, BCC and Bowen's disease (48) and non-melanotic conditions like acne, photodamaged skin and for photorejuvenation (31).

The introduction of ALA and MAL was a major breakthrough in PDT as it offered shorter cutaneous photosensitivity (27) and higher tumor selectivity (49). Clearance of ALA- and MAL- induced PpIX from the body takes only up to 48 h as compared to Photofrin which has a retention varying from 6 days to 3 months (27). For cutaneous applications, 5-aminolevulinic acid- PDT (ALA-PDT) (Levulan ® Kerastick ™) is approved with blue light (BLU-U® Blue Light Photodynamic Therapy Illuminator, Model 4170) (50), while methyl 5-aminolevulinate- PDT (MAL-PDT) (Metvix ®) is approved with Aktilite CL128 ™, LED based narrow band (630 nm) red light technology device (51).

2.3 Mechanisms of PDT

As described above, when the ALA- or MAL-induced photosensitizer PpIX absorbs light, it reaches an excited state. The excited photosensitizer transfers its energy to the ground level oxygen present in the tissue. This creates singlet oxygen, which can kill tumor cells directly by induction of necrosis and/or apoptosis, can cause vasculature shutdown or immune responses (52). The severity of the tissue responses depends both on the lesion type and the treatment regimen (figure 3).

2.3.1 Apoptosis and necrosis

Apoptosis is a controlled-by-cell suicide that results form irreparable damage suffered by the cells. In most cases apoptosis is dependent on the p53 protein. Most of

neoplastic tissues, however, are characterized by lack of p53 activity, further distinguishing them from the surrounding normal tissues. PDT has the advantage that it can lead to p53 independent apoptosis (53;54), which is a "tidy" mechanism that does not result in inflammation.

Necrosis results in cellular membrane breakage and leakage of the cell content into the extracellular space, inducing immune responses and inflammation, which is also observed after PDT. *In vitro* studies demonstrate that the mode of cell death depends on the extent of initial cell damage (55;56)

2.3.2 Immune responses

PDT effects on the immune system can be either immunosuppressive or immunostimulatory (52). Cells dead through necrosis attract leukocytes into the tumor site and increase antigen presentation (52). The acute inflammatory response induced by PDT attracts dendritic cells, which phagocyte the destroyed cells, mature under the influence of locally released cytokines and present the antigen to naive T cells in the local lymph nodes. This leads to maturation of T cells, which subsequently become effector T cells, migrate to the tumor site and kill the tumor cells (figure 3). At this point the tumor environment can be described as a site of chronic inflammation (57). In 1994, Canti *et al.* (58) showed that mice with fibrosarcomas treated with either PDT with aluminum disulfonated phthalocyanine (AIS2Pc) or surgery, had the same survival rate. However, when re-challenged with the fibrosarcomas, only the mice treated with PDT in the first place survived. Subsequent exposure of the surviving mice to another type of cancer proved lethal. This excellent study showed that next to short-term tumor ablation, PDT can induce long-term, specific anti-tumor immunity.

Paradoxically, PDT can also induce immunosupression. PDT with shallow penetrating blue light acts via interleukin 4 (IL4) activation while PDT involving deep penetrating red light acts via the activation of interleukin 10 (IL10) (52). Both pathways lead to short term (up to 28 days) immunosupression. In contrast to UV, surgery, radiotherapy and chemotherapy which suppresses both short- and long-term responses, PDT does not induce long-term immunosupression (51).

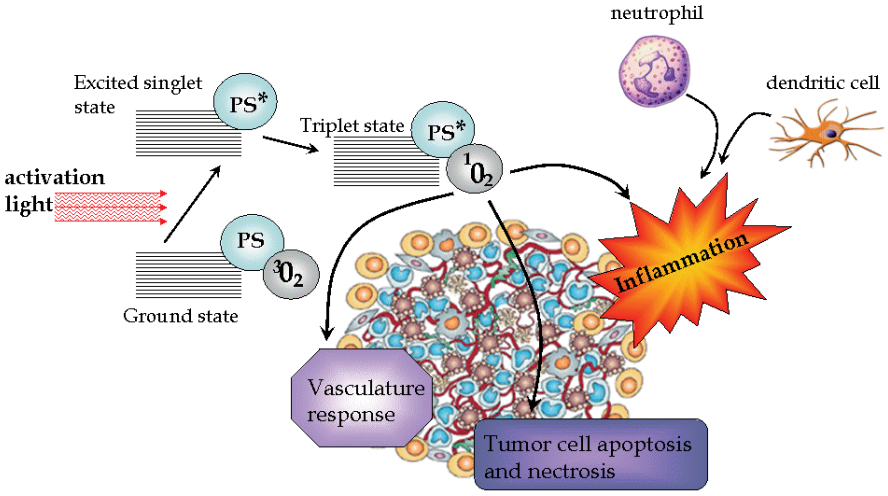


Figure 3. The action mechanism of PDT on tumors. The excited photosensitizer (PS*) transfers its energy onto the molecular oxygen ($^3O_2$) in its proximity. The singlet oxygen ($^1O_2$) reacts with biomolecules and leads to damage that can cause tumor cell death by apoptosis and/or necrosis; microvasculature responses or inflammation. Adapted from Castano *et al*. (52)

2.3.3 Vascular responses

Vascular responses occur immediately after PDT as a result of direct vasculature damage and/or nitric oxide mediated vasoconstriction /vasodilatation, release of vasoactive substances or edema (40;59;60). Vascular damage leads to hypoxia through nitric oxide inhibition and a subsequent contraction of smooth muscle cells surrounding the endothelium of blood vessels (61). Nitric oxide inhibition results also from the PDT oxygen consumption, as nitric oxide is biosynthesized endogenously from L-arginine, oxygen and NADPH by various nitric oxide synthase (NOS) enzymes (61;62). There seems to be a difference in response between normal and tumor microvessels, as normal vessels in the margin of the tumor have a better control over the smooth muscle cells than do tumor vessels (60). On the other hand, a large increase in tissue oxygenation (from 3 up to 9.5 mmHg) after ALA-PDT was reported by Pogue *et al*. (63). The oxygenation returned to baseline levels after 48 h after PDT. The authors explained the results by a decrease in local oxygen metabolic consumption due to cellular damage (63).

The tissue oxygen status during light exposure is an indication of tumor hypoxia, PpIX photobleaching (i.e. the therapy effectiveness) and, possibly, PDT-induced pain.

2.4 PDT-induced pain

2.4.1 Factors influencing pain

Pain during topical PDT occurs during light exposure is experienced to varying degree by the majority of patients and may last for some hours (usually it disappears on the same day) (31;32;64-67). A number of different factors influence the pain during topical PDT, such as the localization, size and the type of lesion, PpIX amount, its localization, the type of PpIX precursor, fluence, fluence rate, fractionated light delivery, light source, wavelength, temperature increase, skin type, patient's age and sex (figure 4). The influence of some of them has neither been clearly confirmed nor disproved as the available knowledge is conflicting.

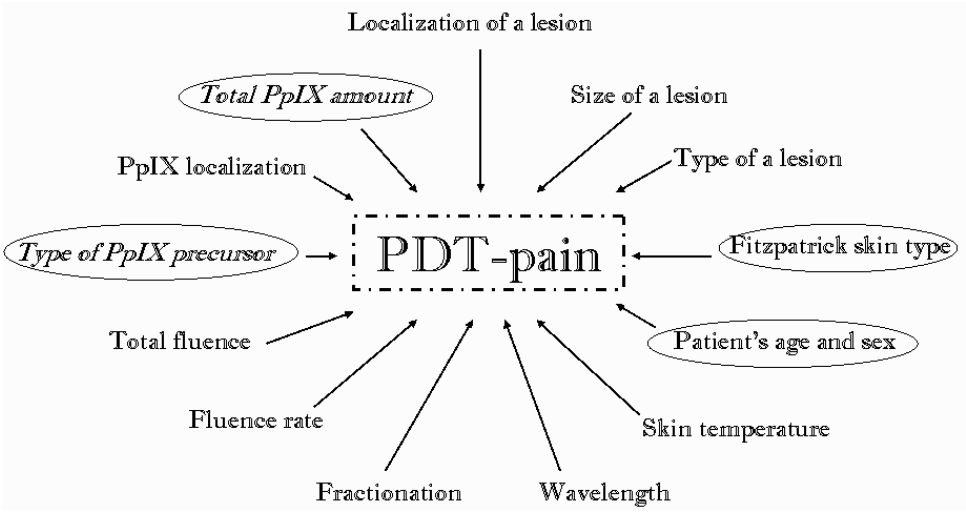


Figure 4. Factors influencing PDT-induced pain. The influence of encircled factors is not confirmed as the existing evidence is conflicting.

2.4.1.1. Total PpIX amount

Light exposure of PpIX leads to creation of $^1O_2$, which in turn leads to cell damage and, consequently, cell death. A correlation between the intensity of pain and PpIX amount in the tissue (68), as well as a correlation between the intensity of pain and photobleaching rate has been demonstrated (43). However, not all studies report such a correlation

(45;64;69). Other factors, intrinsic to the treated disease, such as the type and localization of a lesion, were reported co-determinants of pain with the PpIX levels (43;70;71).

2.4.1.2. Type of PpIX precursors (ALA and MAL)

Different precursors of PpIX may act differently with regard to their uptake by cells, penetration depth, intra-tissue localization of PpIX production, retention time, systemic toxicity, etc. Therefore, it is of interest to investigate the differences between the ALA-PpIX-induced pain and MAL-PpIX-induced pain. Here again, conflicting evidence has been found. ALA is transported through different pathways than ALA esters. In addition it has been suggested that long-chain ALA esters are taken up more efficiently than ALA, possibly by passive diffusion (44). Rud *et al* (44) showed that transport of ALA is Na^+, Cl^- and energy-dependent. These data, together with the fact that ALA lowers blood pressure, just like GABA, point to GABA receptors as possible transporters of ALA into cells (72). Since GABA transporters are found in peripheral neurons, the uptake of ALA, but not MAL, could explain more pain sensations during ALA-PDT than MAL-PDT. Neoplastic cells, due to their high metabolism, have increased demands for amino acids, thus expressing a higher number of receptors. This leads to selective uptake of ALA by neoplastic tissue and the well known selectivity of ALA-PDT. However, when investigating the pain sensations during ALA- and MAL-PDT with the same PpIX levels, no difference was found (43). These findings point back to the importance of the total PpIX amount in the induction of PDT-related pain.

2.4.1.3. Localization, size and type of lesion

There is consensus in the literature that localization, size and type of lesion influence the severity of pain during PDT (66;69;73;74). Treatment of well-innervated areas, like face, hands and perineal region results in more pain than treatment of other regions (69). As for the type of lesion, PDT of psoriasis results in the highest pain scores, followed by actinic keratosis, while BCC seems to be relatively mild in pain (73). Possibly, this is due to different levels of keratinization resulting in different PpXI accumulation patterns in the lesions (75). Treatment of multiple lesions or large areas also results in more intense PDT-pain (66). The increase in pain with increase in the treated area may be explained by the increase in total malignant cells present in the treatment area (69). However, a study by von Oosten *et al*. demonstrated no difference in pain between different sized lesions, nor between lesions differently located (74).

2.4.1.4. Temperature increase

Skin temperature increases during light exposure (70;76), and may influence the severity of experienced discomfort. Since patients characterize the pain as a burning sensation, it has been concluded that it is the absorbed energy delivered by light exposure that leads to local temperature increase (40). Tumors are more sensitive to temperature increase above 41°C than normal, surrounding tissue (77). Warloe *et al.* showed an increase in temperature of skin surface exposed to light at 630 nm with fluence rate of 100-150 mW/cm^2 up to 40°C (76). However, the temperature increase from the increased blood perfusion has not been differentiated. Another study showed that increase in skin temperature above 40°C is possible only when associated with fluence rates higher than 150 mW/cm^2 (70). In clinical applications, fluence rates higher than 75 mW/ cm^2 are rarely used. Thus, the light exposure could not be the sole reason for the pain.

2.4.1.5. Fluence and fluence rate

The fluence rate and total delivered dose influence pain induction and its intensity (68;78;79). Cottrell *et al*. suggested a pain threshold at 60 mW/cm^2, below which the majority of discomfort was alleviated (78). Light exposure at low fluences (9 J/cm^2) prevented fast oxygen depletion and immediate immune response resulting in vasoconstriction, which would impair continuous oxygen supply. After bleaching of 80% PpIX with low fluence rates, an increase in fluence rate did not result in pain increase and allowed for delivery of full treatment dose (78). Recently, a study combining low fluence rates and a fractionated regimen demonstrated that low fluence rates result in more efficient use of the available oxygen, i.e. increased photobleaching rate (80). Therefore, the lower the fluence rate, the higher the photobleaching efficiency with a theoretical maximum below 1 mW/cm^2 (80;81). However, lowering of the fluence rate results in prolonged treatment, and thus may be of limited clinical application value.

2.4.1.6. Fractionated light delivery

Introducing a dark period in the PDT treatment regimen has been shown to give additional PpIX synthesis from still available ALA (82-84). Additionally, fractionation enables re-oxygenation of the treated area, thereby increasing the effect of PDT (85). In 2006, de Haas *et al*. (83) showed that a 2-hours dark period between exposures of 20 and 80 J/cm^2 increased the efficiency of ALA-PDT in BCC patients up to 97%. In 2007, Lindeburg *et al*. concluded that in patients with AK and BCC given Metvix-PDT with two light

exposure fractions, the second one was significantly more painful and became the therapy limiting factor (86).

2.4.1.7. Fitzpatrick skin type

There is a limited number of studies of a possible influence of skin type on the severity of PDT-related pain, and the data are conflicting. A recent study showed a statistical analysis of factors predictive for pain during ALA- and MAL-PDT from over 100 patients (64). The results of this study showed that Bowen's disease (BD) and AK patients with skin types I and II reported higher pain scores than patients with skin type III. Another study on 60 patients diagnosed with BCC, BD and AK reported no correlation between severity of pain and skin type (69).

2.4.1.8. Wavelength

The choice of wavelength for topical PDT should be made by taking into account the thickness of the treated lesion and the absorption characteristics of the tissue to be destroyed (87). Green light (543-548 nm) has been shown to be equally effective and less painful than red light in ALA-PDT in patients with multiple solar keratoses (88). Also full spectrum visible light at high light doses proved effective for ALA-PDT treatment of solar keratoses (89). Another study showed that green light, although less painful, was also less effective than red light (630 +/- 15 nm) in ALA-PDT of Bowen's disease (90). MAL-PDT of AK with red or blue light showed no difference in effectiveness nor in pain (91). Combined violet and subsequently red light showed positive results in melanotic melanomas, where violet light was used to bleach some of the melanin from above the melanomas before commencing red light PDT (92).

2.4.1.9. Patient's sex and age

Male patients seem to experience more pain during ALA-PDT than females (93). However, a number of other studies did not confirm this finding (64;69;74;94). There is agreement in the literature that the patient's age has no significant influence on the experienced level of pain (64;69;74;93;94).

2.4.2 Possible mechanisms of PDT-induced pain

The mechanisms of PDT-induced pain remain largely unknown. However, some theories exist that can help explain certain features of this unpleasant sensation.

2.4.2.1 Peripheral nerve stimulation

The pain appearance is often described as neurogenic, i.e. associated with peripheral nerve stimulation (95;96). A study on a canine model showed a correlation between the ALA-PDT-induced peripheral nerve injury and light dose (97). Generally, pain is believed to be mediated through the non-myelinated C-fibres (figure 5), involving substance P and other neurotransmitters (98). However, desensitization of nerve endings by depleting substance P and other neurotransmitters by repeated contact with capsaicin (chili pepper) during ALA-PDT of AK patients showed no pain-relieving effects (98). Moreover, no substance P was detected in relation to ALA- and MAL-PDT in healthy volunteers (71).

2.4.2.2 GABA receptors

Peripheral nerve stimulation theory may be in line with, previously mentioned, transport of ALA by GABA receptors into the nerve endings (45), which upon light exposure become activated. This does not explain the MAL-PDT induced pain, since MAL is not transported into the nerve endings. On the other hand, when taking into account ALA- and MAL-induced PpIX fluorescence patterns, it shows that ALA-PpIX displays a uniform fluorescence over the applied area, while MAL-PpIX appears in discrete spots (66). It is logical to assume that those spots are hair follicles and sebaceous glands where PpIX is located. The close proximity of nerve endings innervating the hair follicles to the place of photodynamic action may be an explanation for MAL-PDT induced pain (figure 5).

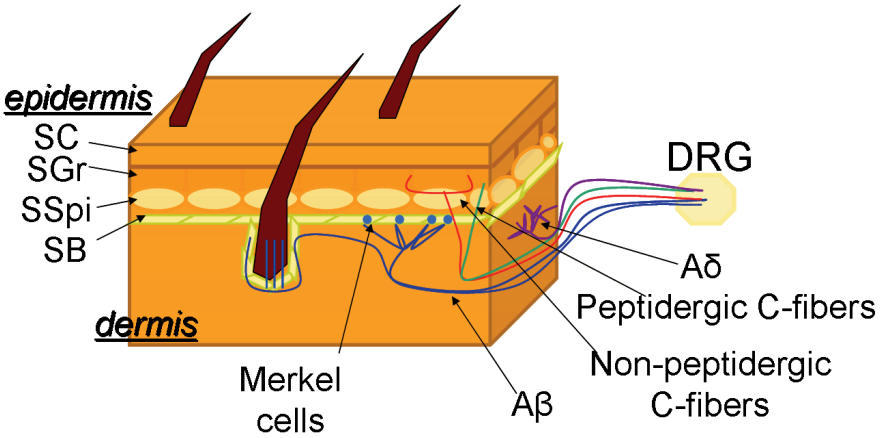


Figure 5. In skin there are 3 types of terminals of primary sensory afferent neurons: 1) Myelinated Aβ fibers which respond to low-threshold, non-painful stimuli, e.g. touch. 2) Aδ fibers, with thin myelination, respond to both non-painful and painful stimuli and 3) C-fibers, non-myelinated fibers, that respond to high- threshold painful stimuli. There are two subtypes of C-fibers: 1) non-peptidergic, localized in *stratum granulosum*, which respond to mechanical stimuli and 2) peptidergic C-fibers, localized in *stratum spinosum*, responding to

heat pain stimuli. DRG stands for dorsal root ganglion.

2.4.2.3 Release of mediators of inflammation

Even though MAL is not taken up into the nerve endings, reactive oxygen species (ROS) produced during light exposure may cause cell damage and/or degranulation of mast cells leading to release of inflammatory mediators, e.g. histamine (99). Mast cell degranulation products, as well as potassium ions, can activate Aδ and C fibers in the skin (figure 5). Histamine is directly involved in the immediate PDT-pain response (99), while bradykinin, serotonin and prostaglandins are probably involved in the later-occurring inflammation.

2.4.2.4 TRPV1 receptor activation

The capsaicin receptor TRPV1 (transient receptor potential cation channel, subfamily V, member 1) is a non-selective, calcium-permeable cation channel that is expressed on peripheral terminals of small to medium diameter sensory neurons and can be activated by, among others, heat stimuli (100). The skin temperature rise caused by energy absorption by PpIX during ALA-PDT is insufficient for actual tissue damage (76). However, the light energy absorption by melanin is much larger than the energy absorption by PpIX and adds to the temperature increase (40). This can provide an explanation for TRPV1 receptor activation during light exposure during topical PDT.

2.5 Topical PDT-pain management

Many attempts to reduce the PDT associated pain have been made, as summarized in figure 6 and table 2, and outlined in more detail below.

2.5.1 Topical anesthesia

Topical anesthesia was the first method employed for this task (66). Unfortunately, local topical anesthesia with lidocaine, tetracaine, prilocaine, morphine and capsaicin proved to be ineffective in relieving the PDT-induced pain (table 2). The acidic pH of the ALA and MAL creams may counteract the actions of topical anesthetics (66). Possibly there may be a saturation of the skin with ALA and MAL creams preventing the anesthetics from penetrating into the tissue (66). Moreover, local anesthetics block only ion channel-mediated painful stimuli and cannot prevent ion leakage from membranes broken by photodynamic action (70). Anesthetics injected deeper can prevent generation of nerve

signals, since these nerves are not reached by the photodynamic effect and, thus, remain intact (70).

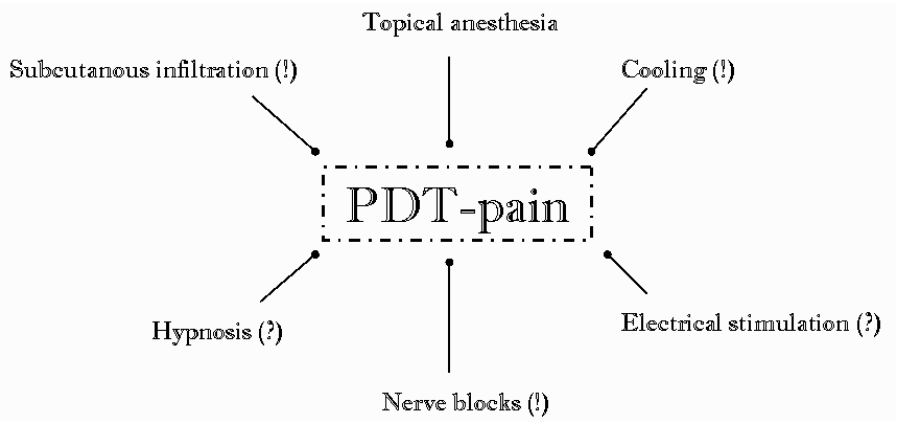


Figure 6. PDT-induced pain management pathways. Exclamation mark (!) follows methods shown to be successful in PDT-pain relief. Question mark (?) stands for methods where the available data are inconclusive. Topical anesthesia is still used for PDT-pain relief, yet the benefit of its use is doubtful.

2.5.2 Electrical nerve stimulation

The mechanism behind the effect of transcutaneous electrical nerve stimulation is based on gate-controlled blocking of nerve fibers in the proximity of the treated area (101). Possible effectiveness of this method has been demonstrated on AK patients during Metvix-PDT (101).

2.5.3 Cooling of the treated area

Cooling with air, water spray or cold packs of the treated site is, next to nerve blocks and subcutaneous injections, the only efficient method for pain relief during light exposure during topical PDT. The reason for its success is not known. However, there are hypotheses that reduction in temperature lowers the tissue metabolism, thus reducing the effects of injury, and causing vasoconstriction, which in turn limits the inflow of inflammatory mediators (66). Topical cooling increases the threshold for pain stimuli and inhibits activation of pain pathways by stimulating myelinated Aδ fibers (102). Moreover, low temperatures may inhibit the activation of TRPV1 receptors, thus decreasing the pain sensations. Additionally, cooling can activate cold and menthol receptor TRPM8 (transient receptor potential cation channel, subfamily M, member 8) (103). Unfortunately, the effectiveness of cooling in pain reduction, during MAL-PDT of AK, BCC and BD seems to reduce PpIX photobleaching, and thus, PDT efficacy (104).

2.5.4 Nerve blocks

A number of studies showed a benefit of nerve blocks in Metvix-PDT for extensive facial AK (105;106). The nerve block method was reported to be superior to cooling with air for extensive AK treated with Metvix-PDT (107). However, pain relief with nerve blocks presents a risk for potential vessel trauma or nerve injury resulting in paresis (108).

2.5.5 Subcutaneous infiltration

Subcutaneous infiltration anesthesia (SIA) was reported to be effective in alleviating pain during Metvix-PDT treatment of patients with actinic chelitis (109) and ALA-PDT treatment of extensive facial AK (110). SIA is not a preferred method for pain relief in proximity of eyes due to a risk for vision impairing by swelling of the eyelids (110). Moreover, the need for multiple injection sites presents a risk for infections (110).

2.5.6 Hypnosis

Pain relief with the use of hypnosis has been demonstrated for treatment of low back pain (111). Hypnosis for pain reduction during ALA-PDT was evaluated on two AK patients. It was concluded that fear or doubt in its effectiveness can impair the pain relief (70).

Table 2 PDT-related pain management methods and their outcome**.**

Pain management method		Pub. year	Authors	N	Outcome
Topical anesthesia	**EMLA**	2006	Langan and Collins (112)	14	No benefit
	lidocaine	2004	Touma *et al*. (113)	18	Minor benefit
	tetracaine	2003	Holmes *et al*. (95)	42	No benefit
	morphine	2006	Skiveren *et al*. (114)	28	No benefit
	capsaicin	2006	Sandberg *et al*. (98)	91	No benefit
Nerve blocks		2008	Paoli *et al*. (106)	16	Beneficial for motivated patients
Subcutaneous infiltration		2007	Borelli *et al*. (110)	16	
Cooling		2004	Pagliaro *et al*. (115)	26	Beneficial, but may interfere with treatment outcome
Hypnosis		2003	Algermissen *et al*. (70)	2	Use limited to the patients who believe in the effect of hypnosis
Electrical nerve stimulation		2008	Halldin *et al*. (101)	14	Minor, but statistically significant benefit

N stands for the number of patients in the study.

3. General experimental considerations

3.1 Chemicals

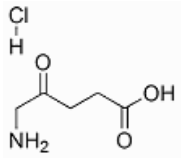
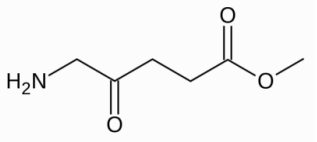
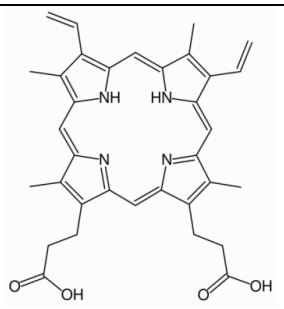
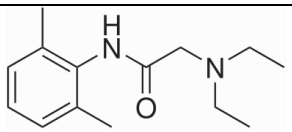
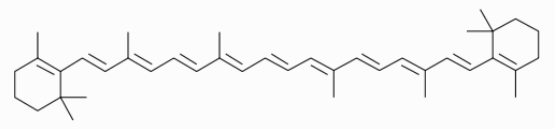
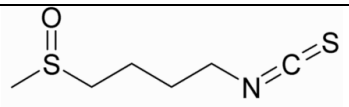
5-aminolevulinic acid hydrochloride (ALA) was used as an active compound in papers I-V. In addition, methyl 5-aminolevulinate hydrochloride (MAL) was used in papers III and IV. Moreover, lidocaine hydrochloride, β-carotene and protoporphyrin IX (PpIX) were used in paper I. PpIX was, together with (R)L-sulforaphane (SF), also used as an active compound in paper II.

In cell work (papers I and II) phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), RPMI medium, penicillin/streptomycin solution, L-glutamine, trypsin/EDTA solution, foetal calf serum (FCS) (paper I) or foetal bovine serum (FBS) (paper II) were used.

For experimental work involving human volunteers (papers III-V), the active compounds were diluted in cream base Unguentum M to reach their final concentration.

Molecular weights and chemical structures of the active compounds are presented in Table 3.

Table 3. Structures and molecular weights of the active compounds used.

Compound	MW (Da)	Chemical structure	
ALA HCl	167.6	$C_5H_{10}ClNO_3$	
MAL	181.6	$C_6H_{11}ClNO_3$	
PpIX	562.7	$C_{34}H_{34}N_4O_4$	
Lidocaine	234.3	$C_{14}H_{22}N_2O$	
β-carotene	536.8	$C_{40}H_{56}$	
SF	177.3	$C_6H_{11}NOS_2$	

3.2 Cells

The human cell lines WiDr (paper I) and A431 (paper II) were used in this work.

The WiDr cell line has been derived from a primary adenocarcinoma of the human rectosigmoid colon of a 78-year-old female in 1978 by Noguci *et al*. (116). This cell line is reprted to have a doubling time of 15 h and a plating efficiency of 51%. Cells attach slowly to the substratum, remain rounded and loosely adherent for 24 to 48 h before flattening into a monolayer (116). The WiDr cell line has a mutation in the gene encoding p53 (117), and after ALA-PDT die only through necrosis (118).

The epidermoid carcinoma cell line A431 was in 1973 derived from a solid tumor from an 85-year-old female. It is one of a series of cell lines established from solid tumors by Giard *et al*. (119) Although the A431 cell line has a mutated p53 gene, it may die also by apoptosis (120).

3.3 Animal work

The part of the work involving an animal (paper III) was approved by the National Animal Authority (Norway) and was performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS). Three separate samples of skin were obtained from one female 12 weeks old NCR nude mouse. The mouse was obtained from The Norwegian Radium Hospital (Oslo, Norway).

During the PpIX fluorescence decay experiment (paper III), which lasted for 3 hours, the mouse was housed alone in the cage in subdued light and was provided with food and water *ad libitum*. The cage was placed in a scantainer providing 22°C and 40-50% humidity. Before i.p. injection of 200 mg/kg ALA in PBS at pH 5, the mouse was anesthetized with Sevoflurane. After 3 hours the mouse was sacrificed by cervical dislocation under Sevoflurane anesthesia.

3.4 Human volunteers

The work involving healthy human volunteers (papers III- V) was approved by the Regional Committee for medical and health research (Regional komité for medisinsk og helsefaglig forskningsetikk, Helseregion Sør, avdeling B, REK Sør B; ref.nr. S-07434b).

The volunteers were a random group of healthy persons. No discrimination of age, gender, religion, skin type or other was made. The test spots were marked on the skin of

the volunteers with a simple non-fluorescent marker and covered with light-impenetrable occlusive dressing. Thus, no restrictions on physical or other activities were made upon the test subjects.

3.5 Light exposure

An in-house built lamp with four fluorescent tubes was used for the *in vitro* studies (papers I and II). The lamp emits light in the region 400–460 nm and with a peak at 420 nm (figure 7). Cells grown in culture dishes were placed on the lamp for light exposure. The fluence rate at the position of the cells was 10 ± 0.5 mW/cm^2 as measured with a photodiode (NewPort, Model 1815-C, Irvine, CA).

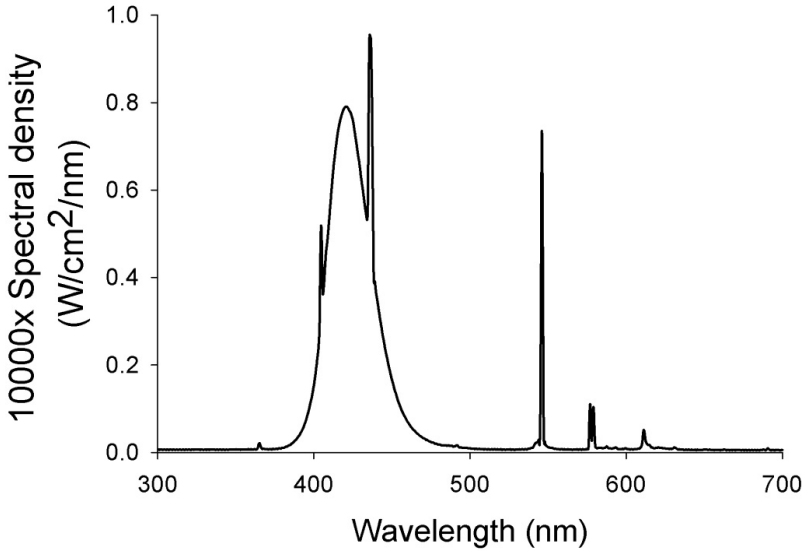


Figure 7 Spectrum of the fluorescent blue lamp used for light exposure of cells (papers I and II). The lamp emits light in the region 400–460 nm with a peak at 420 nm

A red LED (light emitting diodes) lamp was used for the *in vivo* studies (papers II and IV). The lamp emits in the spectral range 580-670 nm, and has a peak at 632 nm (figure 8). The lamp was placed 5 cm above the skin surface. The fluence rate at the surface of the skin was 90 ± 4 mW/cm^2. Similar fluence rates are continually being used in clinical PDT.

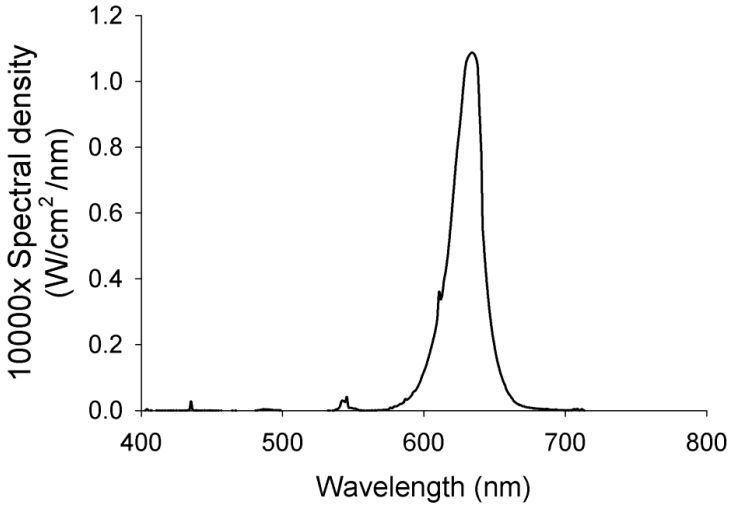


Figure 8 Emission spectrum of the red LED lamp used for exposure of human skin to light (papers II and IV). The lamp emits in the spectral range 580-670 nm, and has a peak at 632 nm

A red laser, λ= 632 nm, (papers III and V) and a violet laser, λ= 405 nm, (paper III) were used in the *in vivo* studies. The violet laser was in-house built consisting of a PMM-608G violet laser diode (405 nm, Photonic Products,UK) which was fitted with an expanding lens and a 20 cm^2 aluminum heat-sink and powered by an adjustable direct current power supply produced in our workshop.

In order to control the violet laser output, a calibration curve was made by measuring the laser output with a PowerMeter photodiode (NewPort, Model 1815-C, Irvine, Calif., USA) as a function of the applied current. The calibration curve is shown in figure 9.

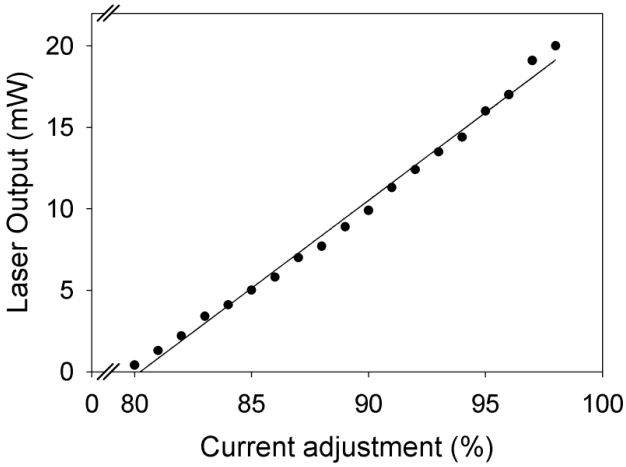


Figure 9 Calibration curve for the in-house built violet laser.

3.6 Pain assessment

Pain was assessed by measuring the exact time (in seconds) from the beginning of light exposure to the onset of pain (paper IV and V). Each volunteer had an additional control spot where cream without active compound was exposed to light and time for pain to occur was measured. Thus, each of the volunteers served as their own control. The volunteers did not know in advance which of the spots contained active compound and which of the spots served as control.

3.7 Reflectance spectroscopy

Erythema index (papers III and IV) was measured using a narrowband reflectance spectrometer DermaSpectrometer®. This instrument applies light from diodes emitting at 568 nm (green) and 655 nm (red). An erythema index is computed from the intensity of the reflected light.

3.8 Fluorescence spectroscopy

The fluorescence of porphyrins produced in the skin was measured in all spots in all volunteers (papers II-V) after cream removal using a fiber-optic probe coupled to a spectrofluorimeter (LS50B, PerkinElmer, Norwalk, CT). Excitation light was set on 407 nm, while the emission spectra were in the range of 560-700 nm, thus ensuring that the major part of the recorded fluorescence is related to PpIX molecules accumulated in the skin.

The PpIX in solutions (papers I and II) as well as cell suspensions (papers I and II) were exposed to light and PpIX fluorescence was measured in cuvettes placed directly in

the standard cuvette holder of a luminescence spectrometer (Perkin Elmer LS45 Norwalk, Conn., USA). The fluorescence background (autofluorescence) of the cells without PpIX was recorded and subtracted from the fluorescence data.

3.9 Statistical analyses

Data are presented as means +/- S.E. (standard error). Experiments in solutions and in cells are shown as means from at least three independent experiments, all done in triplicates. Work performed on volunteers is presented as means of 10 volunteers. The Student's *t*-test was used to evaluate significant differences between data points in cells and solutions. The Student's paired *t*-test was used for comparison of data obtained from volunteers, each volunteer serving as his own control. Values of $p < 0.05$ were considered as indicating significant differences. In all papers calculations were performed using SigmaPlot calculating software. Additionally, in paper V, curve smoothing and artefacts removal was done in MatLab using custom filters.

4. Summary of the publications

4.1 Publication I: The effect of local anesthetic lidocaine on PpIX photobleaching and outcome of ALA-PDT in vitro

Introduction: Lidocaine is used topically to relieve itching, burning and pain during light exposure during topical PDT. It has been reported that lidocaine may act as a singlet oxygen quencher.

Aim: The main goal of this study was to investigate the potential singlet oxygen scavenging properties of lidocaine, as such properties would directly influence the outcome of photodynamic treatment.

Methods: The influence of lidocaine on PpIX monomerization in solution and on PpIX photobleaching in DMSO and in WiDr cells was measured by fluorescence spectroscopy. The effect of lidocaine on PDT outcome was evaluated by measuring the colony- forming ability of the WiDr cells.

Results: Lidocaine concentrations in the range 0.2 - 5 mM increased PpIX fluorescence, possibly by increasing its monomerization. Photobleaching rates of PpIX in solutions were increased in the presence of lidocaine, although not significantly. Lidocaine had no influence on PpIX photobleaching or on PDT efficiency in WiDr cells.

Conclusions: Our *in vitro* study showed no influence of lidocaine on PpIX photobleaching, or on PDT outcome.

4.2 Publication II: Effect of (R)L-sulforaphane on ALA-PDT

Introduction: It has been demonstrated that (R)L-sulforaphane (SF), which is derived from broccoli sprouts, reduces the skin redness and inflammation caused by exposure to ultraviolet (UV) radiation.

Aim: The aim of this investigation was to evaluate the impact of (R)L-sulforaphane (SF) on PpIX production and photobleaching *in vitro,* in solutions and in A431 cells, and *in vivo,* in human skin.

Methods: The influence of SF on PpIX fluorescence and photobleaching in solutions was measured by fluorescence spectroscopy. The dark toxicity of SF in A431 cells, as well as the influence of SF on ALA-PDT outcome, was determined by means of colony assay. PpIX photobleaching in A431 cells and PpIX production after topical application of ALA and /or ALA in conjunction with SF in healthy human skin were measured by fluorescence spectroscopy.

Results: PpIX fluorescence and photobleaching in solution were unaffected by the presence of SF. Neither did SF influence PpIX photobleaching, nor did it influence ALA-PDT outcome in A431 cells in concentrations below 80 μm/L. Above this concentration, SF caused cell detachment from the substratum. SF increased PpIX production in healthy human skin.

Conclusions: (R)L-sulforaphane increased the PpIX production in human skin, but not in A431 cells. It did not influence the PDT outcome. Thus, it presents a safe modality for an anti-erythemal addition to the treatment.

4.3 Publication III: Topical ALA- and MAL- based PDT with red and violet light: influence of wavelength on pain and erythema.

Introduction: PpIX can be activated by light of different wavelengths. Red light penetrates deep into tissue, but is not optimal for PpIX activation. Violet light activates PpIX efficiently, but due to its limited penetration it can be used only for superficial lesions.

Aim: The aim of this study was to determine if light wavelength has influence on pain and erythema induction during topical PDT with ALA and MAL.

*Method*s: Violet and red lasers were calibrated to give the same rate of PpIX photobleaching. The PpIX photobleaching rate was determined by monitoring real-time PpIX fluorescence decay curves in murine skin. ALA- and MAL-PDT were performed on healthy skin of volunteers. Time was measured in seconds from the beginning of light exposure until the onset of pain. Erythema was followed up to 24 h after light exposure by means of reflectance spectroscopy.

Results: No difference in MAL-PDT induced pain and erythema was observed for light of different wavelengths. In the case of ALA-PDT, however, red light induced more pain and longer persisting erythema than violet light.

Conclusions: As far as pain and erythema are concerned, red light induced more side effects than violet light during ALA-PDT. In the case of MAL-PDT, the wavelength did not influence the side effects.

4.4 Publication IV: Microneedle pre-treatment of human skin improves ALA- and MAL-induced PpIX production for topical PDT without increase in pain or erythema.

Introduction: The efficiency of microneedles for transdermal ALA and MAL delivery has been demonstrated in mouse skin. However, mouse and human skin differ significantly.

Aim: This investigation was performed in order to evaluate microneedles as a mechanical means of improving the transdermal drug delivery for topical ALA- and MAL-PDT in human volunteers, as well as to evaluate its impact on the severity of PDT-related side effects.

Methods: PpIX fluorescence in healthy human skin was measured using fluorescence spectroscopy. The transdermal water loss was determined by moisture probe readings of humidity in a chamber attached to the skin of volunteers. Pain was measured in seconds from the beginning of light exposure till the onset of pain. Erythema was monitored for up to 6 h after light exposure using reflectance spectroscopy.

Results: Microneedles were effective in improving the transdermal drug delivery, which resulted in increased PpIX fluorescence after 4 h incubation, both with ALA and MAL, in concentrations below 16%. It did not improve the PpIX production after application of 16% ALA and MAL. The use of microneedles did not increase the severity of pain or erythema.

Conclusions: The use of microneedles allows for reduction in prodrug concentration and incubation time without loss of effectiveness or increase in the severity of PDT-induced side effects.

4.5 Publication V: Bioimpedance for pain monitoring during cutaneous PDT.

Introduction: Electrical impedance spectroscopy is widely used to characterize the electrical properties of a number of human and animal tissues.

Aim: The purpose of this study was to evaluate the usefulness of bioimpedance spectroscopy as a tool for recording real-time PDT-induced changes in the skin impedance related to pain appearance during light exposure.

Methods: The biompedance measurements were taken with a four-electrode set up, during topical photodynamic treatment of healthy human volunteers at frequencies of 10 Hz and 100 kHz. ALA-PpIX production was monitored with fluorescence spectroscopy.

Results: A significant drop in skin impedance both at low and high frequencies was observed from the moment of pain occurrence. The decrease was steeper at the low frequency than at the high frequency. A correlation between the total PpIX fluorescence and the occurrence of pain was observed, as well as a correlation between the absolute bioimpedance drop and the time to pain onset.

Conclusions: Bioimpedance measurements are a relevant tool for monitoring real-time changes in human skin. The PDT-induced pain may originate mostly, but not entirely, in the extracellular compartment and perhaps require a time-delay, possibly a threshold, for occurrence.

5. Discussion

Topical PDT is an effective treatment, and the only limiting factors are the shallow penetration depth of ALA and MAL, the pain and the erythema. Dealing with the pain associated with cutaneous PDT is a delicate balance between the reduction of discomfort and the risk of reduction of PDT efficiency.

As of February 2011, a search of 228 clinical trials registered on the www.clinicaltrials.gov using the key words "photodynamic therapy" and/or "PDT" listed only 7 trials examining PDT-related pain as a primary outcome measure, out of which 3 were in the completing phase. This number is higher than in 2008, when there was only one out of 181 trials listing pain as one of its primary outcome measures. Although, clearly, the interest in this problem is growing, more research into the causation and control of the pain is needed.

5. 1 Control of pain during light exposure during PDT

Many factors influence PDT-induced pain (table 4), and many possible mechanisms may be involved. However, its pathways remain elusive.

5.1.1 Topical anesthesia

Topical anesthetics, including lidocaine, are used in clinical PDT to minimize pain (see section 2.5.1 and table 2). Lidocaine works by blocking the fast voltage gated sodium (Na+) channels in cell membranes (121). Emla® cream (lignocaine 2,5% and prilocaine 2,5%) has been investigated on AK patients, but was ineffective for pain relief during topical PDT (112). On the other hand, Touma *et al*. reported a slight reduction in ALA-PDT-induced pain after application of 3% lidocaine hydrochloride cream on AK patients (113).
Das and Misra (122) reported that lidocaine has singlet oxygen scavenging properties. Since the effectiveness of PDT depends on singlet oxygen, it was of interest to investigate the influence of lidocaine on PDT efficiency. We performed an *in vitro* study on human adenocarcinoma cells and in solutions (paper I). No singlet oxygen scavenging properties of lidocaine were observed under our conditions. The distance between the intracellular localization of lidocaine and the sites of photodynamic action might be too large for singlet oxygen scavenging to occur (paper I). This may indicate why topical anesthetics, in general, seem to have little effect on PDT-induced pain.

5.1.2 Excitation wavelength

The role of the excitation wavelength on the pain induction was studied. Photobleaching of PpIX during light exposure is an indicator of molecular oxygen consumption, and, thus, PDT efficiency (123). According to its absorption spectrum, PpIX can be excited by light of different wavelengths with different penetration depths (figure 2). Even light from computer/tv monitors emitting in red-green-blue, may potentially, activate ALA-PpIX (124). Optimum activation, however, is achieved with blue light at 407-420 nm (125). Unfortunately, blue light does not penetrate deep into the skin. Red light penetrates deeper but excites PpIX less efficiently. Only a limited number of publications have evaluated the use of different wavelengths with regard to PDT-induced pain. Red light was more painful than green light in the treatment of solar keratosis (88) and Bowen's disease (90). In our study (paper III), we used an innovative method for direct comparison of violet and red light. Both ALA and MAL were used to see if the up-take mechanism and/or the PpIX localization have any influence. Topical ALA-PDT with blue light required longer exposure times to induce pain and gave less erythema as compared with topical ALA-PDT with red light. For MAL-PDT such differences were not observed. These findings are in agreement with a recent publication on MAL-PDT of photodamaged skin with blue and red light, where no difference in treatment outcome and/or side effects was found (91).
In the case of superficial lesions, the use of violet light with either MAL or ALA is beneficial as it provides good treatment outcomes and low pain levels. For thick lesions, deeper penetrating red light, with either ALA or high concentrations of MAL, is recommended as it ensures good treatment outcomes. However, the pain is likely to be larger than with violet light.

5.1.3 PpIX level

The influence of the PpIX concentration on the severity of PDT-induced pain is an unresolved question. A positive correlation between PDT-induced pain and PpIX level was observed in AK patients treated with MAL-PDT (68). No correlation between PpIX level and pain was seen in healthy human volunteers during ALA-PDT (45) and in AK, BCC and BD patients treated with ALA-PDT (64;69) and MAL-PDT (64). Moreover, a difference in pain experience between BD and BCC patients was reported when the PpIX levels were similar (43), suggesting that factors other than PpIX level, e.g. PpIX distribution, are important. Algermissen *et al*. (70) suggested that it is the PpIX distribution in the lesion and not the intensity of the PpIX fluorescence that determines the intensity of pain. All of the

above-mentioned studies employed one of the numeric rating scales (pain intensity numeric rating scale (PI-NRS), visual analogue scale (VAS) or pain logger device) to determine the intensity of the observed pain. The numeric rating scales range from 0 (= no pain) to 10 (= worst imaginable pain) where patients are asked to rate their discomfort during (pain logger device) or after the illumination (VAS, PI-NRS).

5.2 Pain assessment

In our studies we used a novel approach for measuring the PDT-induced pain: we measured time in seconds from the beginning of the light exposure till the onset of pain. This approach brings a new dimension to the pain assessment, since it is easier to determine whether or not one feels pain, than to describe the level of felt discomfort on a numerical scale, often as a recollection after the end of PDT session. This method is reliable and reproducible since every volunteer serves as his own control. The time for pain to occur on the test spots is measured against the time for pain to occur on control spots on the same person thus, eliminating the problem of different personal pain thresholds.

The results of our study (paper IV) on 10 healthy volunteers, during ALA-PDT, show a correlation between PpIX levels and induction of the pain. Considering that the majority of studies using numeric methods for pain assessment report no correlation between PpIX level and pain, and that our method for pain determination reflects the time-course of the onset of pain, it is probable that the PpIX level is more predictive of the length of the pain sensation rather than the intensity of it.

5.3 The influence of skin penetration enhancers on pain

One of the rate-limiting factors in the production of ALA-induced PpIX is the penetration of ALA through the *stratum corneum* and then through the plasma membrane of neoplastic cells. In order to increase the penetration depth of ALA into tissue, several methods have been proposed, such as different formulations with penetration enhancers (e.g. DMSO, EDTA, azone, glycolic acid or oleic acid) (126), mechanical methods (curettage, ultrasound, iontophoresis, electroporation and electrophoresis) (127), and chemical derivatization of ALA (23;38).The use of chemical substances as penetration enhancers has to be well balanced as they may potentially interact with the applied prodrug (128), result in skin saturation, thus reducing prodrug penetration (66) or influence the important oxygen supply (129). Mechanical methods for improving drug penetration into skin include removal of *stratum corneum* by tape stripping, curettage, debulking, microdermabration or

laser ablation (126). However, these techniques, although effective, are often painful and lead to bleeding and/or crusting (130).

The use of microneedles for mechanical penetration enhancement proved to be successful and did not evoke additional side effects (paper IV). *Stratum corneum* is difficult to penetrate for both ALA and MAL, and since our studies were performed on healthy skin of the volunteers, 24 hours application times were used to ensure that an amount of prodrug sufficient for photosensitization would penetrate through the *stratum corneum* barrier.

The applied microneedles were microscopic polymer spikes attached to a square base (figure 1, paper IV).The base ensured that the microneedles did not penetrate deeper than to a pre-determined depth (~ 600 μm). The application of microneedles was judged by the volunteers as painless. It increased the skin permeation for up to two hours (as can be concluded from transepidermal water loss measurements, paper IV, figure 5). Our use of healthy human skin distinguishes this study from others. Although the use of microneedles for improvement of transepidermal drug delivery is not a new concept, previous studies were carried out on mouse skin (131), which differs significantly from human skin by being much thinner. The obtained conclusions therefore can not be directly transferred into clinical settings (132). Our results (paper IV) demonstrate the effectiveness of microneedles as a mechanical improvement of transdermal delivery of ALA and MAL after short incubation times (4 h). In addition, application of microneedles was painless, did not increase the PDT-related pain or erythema. Moreover, a possible saturation of the heme biosynthesis pathway was reached, thus allowing reduction of ALA and MAL concentrations, resulting in potential decrease of costs per patient.

These findings support a new trend emerging in the field of PDT. PDT needs to be efficient, patient- and clinician-friendly by minimizing cost and time, and recently, also ambulatory (133;134).

5.3 PDT-induced erythema

Although many authors mention erythema as one of the most common side effects of topical PDT (35;71;124), there are very few studies directly measuring/monitoring PDT-induced erythema. In 2004, Clark *et al*. described erythemal response after topical ALA-PDT in 10 healthy subjects (135). Their results showed a peak of erythema within 1-2 h after light exposure. Our results, however, do not support this finding. The erythemal response observed in our studies peaked either immediately after the light exposure (paper IV) or within half an hour (paper III). The reason for these discrepancies may be differences

between the site of measurements (back and lower leg vs forearm), or well known interpersonal variations between volunteers (136). Since Clark *et al*. did not find any correlation between the extent of erythema and treatment site, nor between erythema and types of light sources used (135), the reason for the discrepancy seems to be interpersonal variations.

Attempts to reduce erythema with natural compounds, such as (R)L-sulforaphane (SF) derived from broccoli sprouts and other cruciferous vegetables, are safe. However, since introducing new chemical compounds into the course of treatment may affect the PDT outcome, we investigated the influence of SF addition on ALA-PpIX production and bleaching in solutions, cells (A431 cell line) and human skin (paper II). To our surprise, adding SF not only maintained the effectiveness of PpIX photobleaching, but increased the PpIX production in human skin. SF was chosen for our study due to its anti-erythemal properties, although it has also been shown to enhance detoxification of carcinogens (137), block chemically induced carcinogenesis in animals (138), inhibit tumor growth (139), arrest cell cycle progression and enhance apoptosis (140). Our data, together with all these findings strongly suggest beneficial effects of incorporating SF into the course of PDT treatment.

5.4 Real-time PDT measurements

Bioimpedance measurements of human skin during light exposure revealed a time delay in pain sensation occurrence (paper V). This delay can be explained by a threshold of damage that has to be reached before pain occurs. Thus, an increase in temperature during light exposure may be needed to activate TRPV1 channels (capsaicin receptors) that lead to nerve responses (100), or there may be a time delay needed between hydrogen peroxide (H_2O_2)-induced mast cells degranulation and histamine release which leads to activation of C-fibers (141).

A duality of the PDT-induced pain was documented by Lipson and Baldes (19), Holmes *et al*. (95), and recently, by us (paper V). The first PDT-pain component is an early sensation of stinging - possibly neurogenic. The following burning sensation is a late, inflammatory process. The biompedance measurements at low frequency at about 7 min. of light exposure (paper V) revealed a plateau, which may reflect a shift from vasoconstriction (the narrowing of blood vessels) to vasodilatation (the widening of blood vessels) of small vessels; or a change in the tissue response from early, neurogenic, to late, inflammatory, reactions. This observation is in agreement with the reports from Phase III clinical trials on ALA-PDT treatment of nonhyperkeratotic actinic keratoses of the face and scalp on 243

subjects, where the peak of stinging and burning was observed approximately 6 min into the light exposure (35). Although an exact explanation remains speculative, the current state of knowledge may be summarized as follows. PDT-induced pain response can be divided into an early and a late phase (figure 10). Early response starts with the light activation of PpIX (hv-excitation light) that leads to 1) production of reactive oxygen species, including singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), 2) damage to neoplastic cells and/or nerve cells resulting in leakage of their content into the extracellular space, and 3) degranulation of mast cells and release of, among others, histamine. The length of the early response can vary from few seconds to few minutes depending on PpIX concentration, localization, distribution inside the lesion and other parameters shown in table 4. The pain signal is possibly triggered by an unknown mediator/change taking place mainly in the extracellular compartments of the tissue. Whether the strength of the pain signal influences the extent of the inflammation is not known. The late response starts with the release of mediators of inflammation from degranulated mast cells and/or damaged cells.

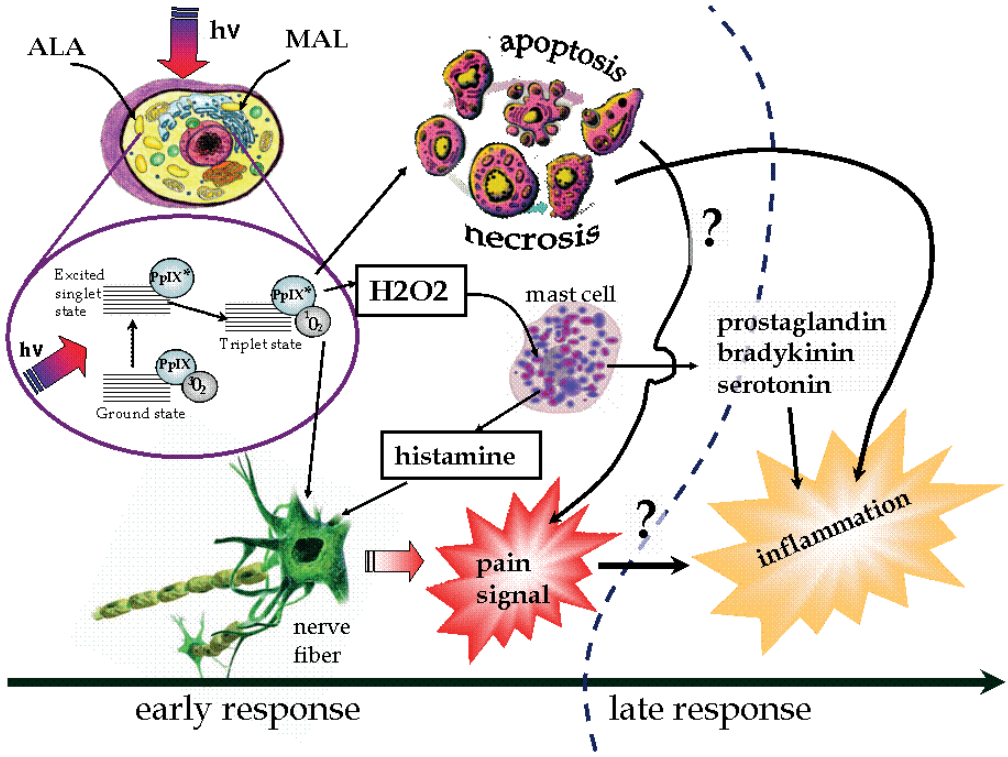


Figure 10. Possible PDT-induced pain mechanisms. See text for details.

Study	N	Localization of lesion(s)	Diagnosis	Prodrug	Dose (fluence), fluence rate	Light source	Influence on pain
Total protoporphyrin IX level							
Valentine et.al. 2011	40	Various localizations	BCC and BD	ALA and Metvix	75 J/cm^2 80 mW/cm^2	Aktilite ®	Factors other than PpIX level matter more for pain prediction
Mikolajewska et al. 2011	10	Healthy volunteers	Healthy volunteers	ALA	300 mW/cm^2	red laser (632 nm)	A positive correlation between PpIX level and pain
Wiegell et al. 2008	26	Different localizations	AK	MAL	37 J/cm^2	Red LED (632nm)	A positive correlation between PpIX level and pain
Langan et al. 2005	14	Scalp	AK	ALA	30 J/cm^2 30 mW/cm^2	PDT 1200 L	No correlation between pain and PpIX level.
Wiegell et al. 2003	20	Healthy volunteers	Healthy volunteers	ALA and MAL	70 J/cm^2 90 mW/cm^2	CureLight 570-670nm	No correlation between pain and PpIX level.
Grapengiesser et al. 2002	60	Various localizations	AK, BCC, BD	ALA	50-130 J/cm^2 76.5 mW/cm^2	PDT 1200L	No correlation between pain and total PpIX level.
Light fractionation							
Wiegell et al. 2008	26	Different localizations	AK	MAL	37 J/cm^2 34mW/cm^2 and 68mW/cm^2	Red LED (632nm)	No difference in pain between first and second treatment
Lindeburg et al. 2007	38	Various localizations	AK, BCC and rosacea	Metvix	37 J/cm^2	CureLight (632nm)	2nd treatment was more painful than 1st
De Haas et al. 2006	154	Various localizations	BCC	ALA	75 J/cm^2 20 J/cm^2 80 J/cm^2 50mW/cm^2	Laser 630nm Medeikonos 590-650 nm LED 633nm	Fractionation increases PDT outcome.

Fitzpatick skin type							
Grapengiesser et al. 2002	60	Various localizations	AK, BCC, BD	ALA	50-130 J/cm² 76.5 mW/cm²	PDT 1200L	No correlation between pain and FST.
Virgili et al. 2010	121	Various localizations	AK, BCC	?	?	?	FST III experience more pain than FST IV
Artis et al. 2010	108	Various localizations	BCC, AK and BD	ALA and MAL	100 J/cm² 37 J/cm²	Incoherent halide lamp PDT 1200L Aktilite ®	More pain in FST I and II than in FST III
Patients' age and sex							
Grapengiesser et al. 2002	60	Various localizations	AK, BCC, BD	ALA	50-130 J/cm² 76.5 mW/cm²	PDT 1200L	No correlation between pain and patient's age or sex.
Van Oosten et al. 2006	57	Various localization	BCC	ALA	10-150 J/cm² 40-100 mw/cm² 10-200 J/cm² 10-200 mw/cm²	Medeikonos (580-680 nm) PDT 1200 (600-750 nm)	No difference in pain between men and women.
Artis et al. 2010	108	Various localizations	BCC, AK and BD	ALA and MAL	100 J/cm² 37 J/cm²	Incoherent halide lamp PDT 1200L Aktilite ®	No correlation between patient's age or sex and pain
PpIX precursors (ALA vs MAL)							
Valentine et.al. 2011	40	Various localizations	BCC and BD	ALA and Metvix	75 J/cm² 80 mW/cm²	Aktilite ®	No difference between ALA- and MAL-induced pain
Steinbauer et al. 2009	34	Healthy volunteers	Healthy volunteers	ALA and	75 J/cm² 176 mW/cm²	PDT 1200L (580-740 nm)	No difference between ALA- and

				MAL			MAL-induced pain
Moloney and Collins, 2007	16	Scalp	AK	ALA and MAL	50 J/cm² 50 mW/cm²	PDT 1200 L (580-740 nm)	More pain with ALA than with MAL
Wiegell et al. 2003	20	Healthy volunteers	Healthy volunteers	ALA and MAL	70 J/cm² 90 mW/cm²	CureLight 570-670nm	MAL less painful than ALA
Wiegell and Wulf 2006	15	Face	Acne vulgaris	ALA and MAL	37 J/cm² 34 mW/cm²	Aktlite ®	No difference in pain, more side effects with ALA.
Kasche et al. 2006	69	Scalp	AK	ALA and MAL	100 J/cm² 160 mW/cm²	PDT 1200 L (580-740 nm)	More pain with ALA than with MAL
Kijupers et al. 2006	39	Various localizations	BCC	ALA and MAL	75 J/cm² 100 mW/cm²	PDT 1200 L (580-740 nm)	No difference between ALA and MAL-induced pain
Artis et al. 2010	108	Various localizations	BCC, AK and BD	ALA and MAL	100 J/cm² 37 J/cm²	Incoherent halide lamp: PDT 1200L Aktilite ®	No difference in pain induction between ALA and MAL
Light source and wavelength							
Ericson et al. 2004	37	Head, neck, upper chest	AK	ALA	100 J/cm2 30mW/cm² 45mW/cm² 50mW/cm² 75mW/cm²	580-650nm 580-690nm	Less pain with a non-coherent light source
Morton et al 2000	16	Legs	BD	ALA	Red: 125 J/cm², 86 mW/cm² Green: 62.5 J/cm² 86 mW/cm²	Red 630nm Green 540nm	Red more painful than green
Van Oosten et al. 2006	57	Various localization	BCC	ALA	10-150 J/cm² 40-100 mw/cm²	Medeikonos (580-680 nm)	Medeikonos less painful than PDT

					10-200 J/cm² 10-200 mw/cm²	PDT 1200 (600-750 nm)	1200
Babilas et al. 2007	25	Face	AK	MAL	37 J/cm² 80 J/cm² 50mW/cm²	Red laser, LED 630 nm, VPL 610-950 nm	Pain lowest with VPL
Artis et al. 2010	108	Various localizations	BCC, AK and BD	ALA and MAL	100 J/cm² 37 J/cm²	Incoherent halide lamp PDT 1200L 580-750 nm Aktilite ® 630nm	No correlation between light source, its wavelength and pain
Mikolajewska et al. 2009	10	Healthy volunteers	Healthy volunteers	ALA and MAL	25 J/cm² 100 mW/cm² 2 J/cm² 8 mW/cm²	Red laser (632 nm) violet laser (405 nm)	Red light is more painful than violet with ALA. No difference for MAL.
Fritish et al. 1997		Face	Solar keratoses	ALA		Green 543-548 nm Red 630nm	Green less painful than red
Type, size and localization of the lesion							
Artis et al. 2010	108	Various localizations	BCC, AK and BD	ALA and MAL	100 J/cm² 37 J/cm²	Incoherent halide lamp PDT 1200L Aktilite ®	No correlation between, type, size and localization of the lesion and pain
Van Oosten et al. 2006	57	Various localization	BCC	ALA	10-150 J/cm² 40-100 mw/cm² 10-200 J/cm² 10-200 mw/cm²	Medeikonos (580-680 nm) PDT 1200 (600-750 nm)	No difference in pain with regard to size and place of lesion.
Wiegell et al. 2008	26	Various localizations	AK	MAL	37 J/cm²	Red LED (632nm)	Most pain on face and scalp
Fluence and fluence rate							
Cottrell et al. 2008	26	Various localizations	BCC	ALA	200 J/cm² 10mW/cm²	Red laser 632.8nm	Lower pain with lower fluence rate

					20mW/cm^2 40mW/cm^2 50mW/cm^2 60mW/cm^2 150mW/cm^2		
Radakovic-Fijan et al. 2005	27	Scalp, face	AK	ALA	70 J/cm^2 100 J/cm^2 140 J/cm^2 100mW/cm^2	Red lamp 600-740nm	Higher dose resulted in more pain
Radakovic-Fijan et al. 2005	29	Trunk, arms, legs	Psoriasis	ALA	5 J/cm^2 10 J/cm^2 20 J/cm2 60mW/cm^2	Red lamp 600-740nm	Pain increased with higher dose
Horfelt et al 2007	15	Face, back	Acna vulgaris	ALA	30 J/cm^2 50 J/cm^2 70 J/cm^2 50mW/cm^2	Red lamp 600-740nm	Pain increased with higher dose
Clark et al. 2003	207	Various localizations	BD, BCC, AK, warts	ALA	150 J/cm^2 20-25 mW/cm^2 125 J/cm^2 80-150 mW/cm^2	Red lamp 570-680nm, laser 630nm	Efficacy is similar for broadband and laser. Higher fluence- more pain
Ericson et al. 2004	37	Head, neck , upper chest	AK	ALA	100 J/cm2 30mW/cm^2 45mW/cm^2 50mW/cm^2 75mW/cm^2	580-650nm 580-690nm	High fluence rate resulted in more pain than low fluence rate
Wiegell et al. 2008	26	Different localizations	AK	MAL	37 J/cm^2 34mW/cm^2 and 68mW/cm^2	Red LED (632nm)	High fluence rate resulted in more pain than low one

Table 4. Factors influencing PDT-induced pain. N – number of volunteers/ patients; PS-photosensitizer precursor; AK- actinic keratosis; BCC- basal cell carcinoma; BD- Bowen's disease

5. Conclusions

Pain is strongly individual, difficult to quantify, describe and, most of all, compare. The goal of this thesis was to investigate the influence of factors that may be used to reduce the pain on the treatment efficiency (lidocaine and (R)L-sulforaphane), as well as possible influences on pain sensations of factors potentially modulating the treatment (violet/red excitation light and microneedles), and finally to shed light on the photodynamic action and pain mechanism through real-time measurements of skin bioimpedance during light exposure. The results of our studies demonstrated that:

1. Lidocaine, although being a singlet oxygen quencher, does not influence the PDT efficiency *in vitro*. Our results suggest a difference in intracellular localization of the lidocaine activity and photodynamic action.
2. The anticancer and anti-erythemal drug, SF, increased PDT efficiency by increasing ALA-PpIX production.
3. Violet light should be used with MAL or ALA for superficial lesions, while red light should be used with ALA for thicker lesions.
4. Red light induced more pain than violet during PDT with ALA, while there was no difference in pain during PDT with MAL.
5. The use of microneedles prior to ALA- and MAL-PDT allowed for reduction of the prodrug concentration and application time without increase in side effects.
6. A decrease in skin impedance both at 10 Hz and at 100 kHz may imply that the pain-inducing changes take place mainly in the tissue extracellular compartments.

These findings suggest than careful adjustment of the excitation light wavelength and the type of prodrug to the thickness of the treated lesion, and use of microneedles and SF for improved PpIX production can reduce PDT-induced pain and potentially improve the treatment outcome.

6. Future prospects

The results presented in this thesis show that the initial photodynamic damage probably takes place at a distance from nerve endings. Thus, the resulting pain signal is likely not mediated through Na^+ channels. Moreover, the pain seems to arise faster the deeper the photodynamic action occurs (since deeply penetrating red light is more painful than shallow penetrating violet light). Furthermore, the PpIX intra-tissue distribution seems to play a larger role than the PpIX concentration. All above-mentioned facts point to the importance of the PpIX localization.

The chemical structure of the prodrug influences its penetration and pharmacokinetics (44;142). Moreover, ALA and MAL are taken up into cells by different mechanisms (44), and the uptake of MAL does not inhibit the uptake of ALA (45). Therefore, it would be interesting to investigate a mixture of ALA and MAL with respect to PDT efficiency and sensation of pain.

Furthermore, since ALA and MAL penetrate to different depths, (in contrast to ALA, MAL does not give a systemic effect after topical application (39)) it could be beneficial to expose lesions first to blue light and subsequently to red light. This procedure might cause 'layer- by- layer' killing of cells and bleaching of the PpIX and prevent rapid depletion of oxygen. It might also prevent hypoxia and minimize the pain sensation. Additional application of a vasodilatating drug on the outside margin of the treated lesion (healthy surrounding tissue) could provide an undisturbed oxygen flow, even at high fluence rates.

It seems worthwhile to investigate further the relationship between light intensity and pain. Why do high fluence rates induce pain and not low fluence rates? Light delivery at low fluence rates allows reoxygenation during the light exposure, thus avoiding oxygen depletion and hypoxia. Tromberg *et al*. (143) distinguished three consecutive processes during light exposure during PDT: 1) initial consumption of oxygen and production of singlet oxygen, 2) local hypoxia and 3) ischemia. When molecular oxygen is depleted and the tissue reaches hypoxia, excited PpIX may react with other biomolecules leading to generation of, among others, pain mediators.

We have shown that pain sensation occurs with a time-delay after the start of the light exposure and that the onset of pain is accompanied by a detectable drop in bioimpedance. This time delay raises a range of questions. Is it related to the time needed for skin temperature to rise above the level of thermal nerve activation, or is it needed for

enough damage to the neoplastic cells to occur, resulting in leakage of cell content and beginning of an inflammatory reaction?

Possibly more than one nerve cell membrane must be broken before a big enough leakage of neurotransmitters into the extracellular space occurs, causing activation of neighboring nerve cells that leads to pain signals. Activation of nerve cells by histamine released from degranulated mast cells has been shown to be involved in the early phase of PDT-pain response (99). Mast cells can undergo different types of degranulation processes, some of which involve partial release of the granule matrix of mast cells into the extracellular space (144). Perhaps the time needed for PDT-induced pain to occur reflects the time needed for degranulation of mast cells and release of histamine.

There might also be a yet unknown product of the secondary photochemical reactions ongoing during light exposure in human skin, and the time delay may be needed for the concentration of such a product to become high enough for nerve cell activation/damage to occur. It is not unlikely that such a process could require specific conditions, e.g. a given level of hypoxia.

By identifying the mechanism behind the observed threshold/time delay, we may improve our understanding of PDT-induced pain and ultimately come closer to an efficient way of reducing pain during treatment.

7. References

1. Pervaiz,S. and Olivo,M. (2006): Art and science of photodynamic therapy. *Clin Exp Pharmacol.Physiol.*, 33:551-556.

2. Raab,O. (1900): Ueber die Wirkung fluorescirender Stoffe auf Infusorien. *Z.Biol*, 39:524-546.

3. von Tappeiner,H. and Raab,O. (1900): Ueber die Wirkung fluorescirender Stoffe auf Infusorien nach Versuchen von O. Raab . *Muench Med Wochenschr*, 47:5-7.

4. Babilas,P., Schreml,S., Landthaler,M., and Szeimies,R.M. (2010): Photodynamic therapy in dermatology: state-of-the-art. *Photodermatol Photoimmunol Photomed*, 26:118-132.

5. Jesionek,A. and von Tappeiner,H. (1905): Zur behandlung der Hautcarcinome mit fluorescierenden Stoffen. *Arch Klin Med*, 82:223

6. Moan,J. and Peng,Q. (2003): Review. An outline of the hundred-year history of PDT. *Anticancer Res*, 23:3591-3600.

7. von Tappeiner,H. and Jodlbauer,A. (1994): Über die Wirkung der photodynamischen (fluoreszierenden). Stoffe auf Infusorien. *Dtsch Arch Klin Med*, 80:427-487.

8. Hausmann,W. (1911): The sensitising action of hematoporphyrin. *Biochem Z*, 30:176

9. Granick,S., Bogorad,L., and Jaffe,H. (1953): Hematoporphyrin IX, a probable precursor of protoporphyrin in the biosynthetic chain of heme and chlorophyll. *J Biol Chem*, 202:801-813.

10. Gregorie,H.B.Jr. and Green,J.F. (1965): Hematoporphyrin-derivative fluorescence in malignant neoplasms. *J S C Med Assoc*, 61:157-164.

11. Mack,H.P., Diehl,W.K., Peck,G.C., and Figge,F.H. (1957): Evaluation of the combined effects of hematoporphyrin and radiation. I. Treatment of carcinoma of the cervix. *Cancer*, 10:529-539.

12. Smetana,H. (1928): Studies upon the physiological action of hematoporphyrin. *J Exp Med*, 47:593-610.

13. Ledoux-Lebards, C. Annales de l'institut Pasteur 16, 593. 1902. (GENERIC)
Ref Type: Journal (Full)

14. Meyer-Betz,F. (1913): Untersuchungen uber die Biologische (photodynamische) Wirkung des hamatoporphyrins und anderer Derivative des Blut-und Gallenfarbstoffs. *Dtsch Arch Klin Med*, 112:503

15. Foote,C.S. (1987): Type I and type II mechanisms of photodynamic action. In: *Light-Activated Pesticides*, edited by J.R.Heitz, et al, pp. 22-38. American Chemical Society ,

16. Figge,F.H., Weiland,G.S., and Manganiello,L.O. (1948): Studies on cancer detection and therapy; the affinity of neoplastic, embryonic, and traumatized tissue for porphyrins, metalloporphyrins, and radioactive zinc hematoporphyrin. *Anat Rec*, 101:657

17. Berlin,N.I., Gray,C.H., Neuberger,A., and Scott,J.J. (1954): The metabolism of the porphyrin precursor, delta-aminolaevulic acid, in normal man and in the rat. *Biochem J.*, 58:

18. Schwartz,S.K., Absolon,K., and Vermund,H. (1955): Some relationships of porphyrins, x-rays and tumours. *Univ.Minn.Med.Bull.*, 27:7-8.

19. Lipson,R.L. and Baldes,E.J. (1961): Histamine and Photodynamic Action. *Arch Dermatol*, 83:99-101.

20. Moore,M.R., Thompson,G.G., Goldberg,A., Ippen,H., Seubert,A., and Seubert,S. (1978): The biosynthesis of haem in congenital (erythropoietic) porphyria. *Int J Biochem*, 9:933-938.

21. Kessel,D. (1986): Photosensitization with derivatives of haematoporphyrin. *Int J Radiat Biol Relat Stud Phys Chem Med*, 49:901-907.

22. Malik,Z. and Lugaci,H. (1987): Destruction of erythroleukaemic cells by photoactivation of endogenous porphyrins. *Br J Cancer*, 56:589-595.

23. Peng,Q., Warloe,T., Moan,J., Heyerdahl,H., Steen,H.B., and Giercksky,K.E. (1995): ALA derivative-induced proto- porphyrin IX built-up and distribution in human nodular basal cell carcinoma. *Photochem Photobiol*, 61:82S

24. Mang,T.S. and Wieman,T.J. (1987): Photodynamic therapy in the treatment of pancreatic carcinoma: dihematoporphyrin ether uptake and photobleaching kinetics. *Photochem Photobiol*, 46:853-858.

25. Kennedy,J.C., Pottier,R.H., and Pross,D.C. (1990): Photodynamic therapy with endogenous protoporphyrin IX: basic principles and present clinical experience. *J Photochem Photobiol B.*, 6:143-148.

26. Moan,J. and Berg,K. (1991): The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. *Photochem Photobiol.*, 53:549-553.

27. Bellnier,D.A., Greco,W.R., Loewen,G.M., Nava,H., Oseroff,A.R., and Dougherty,T.J. (2006): Clinical pharmacokinetics of the PDT photosensitizers porfimer sodium (Photofrin), 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (Photochlor) and 5-ALA-induced protoporphyrin IX. *Lasers Surg Med.*, 38:439-444.

28. DUSA Pharmaceuticals, Inc. U.S. FDA approves Levulan for the treatment of actinic keratoses. http://www.dusapharma.com/about-dusa.html . 2011. 22-3-2011. (GENERIC)
Ref Type: Electronic Citation

29. PhotoCure ASA. Metvix receives full reimbursement for premalignant skin cancer and skin cancer in Sweden. http://www.photocure.com/NEWS/newcontainer/Metvix-photodynamic-therapy-receives-full-reimbursement-for-premalignant-skin-cancer-and-skin-cancer-in-Sweden/ . 2011. 22-3-2011. (GENERIC)
Ref Type: Electronic Citation

30. PhotoCure ASA. Metvixia and Aktilite CL128 approved by FDA. http://www.photocure.com/NEWS/newcontainer/Metvixia-and-Aktilite-CL128-approved-by-FDA/ . 2011. 22-3-2011. (GENERIC)
Ref Type: Electronic Citation

31. Christensen,E., Warloe,T., Kroon,S., Funk,J., Helsing,P., Soler,A.M., Stang,H.J., Vatne,O., and Mork,C. (2010): Guidelines for practical use of MAL-PDT in non-melanoma skin cancer. *J Eur Acad.Dermatol Venereol.*, 24:505-512.

32. Morton,C.A., McKenna,K.E., and Rhodes,L.E. (2008): Guidelines for topical photodynamic therapy: update. *Br.J Dermatol.*, 159:1245-1266.

33. Morrow,D.I., Garland,M.J., McCarron,P.A., Woolfson,A.D., and Donnelly,R.F. (2007): Innovative drug delivery strategies for topical photodynamic therapy using porphyrin precursors. *J Environ Pathol Toxicol Oncol*, 26:105-116.

34. Scheindlin,S. (2004): Transdermal drug delivery: PAST, PRESENT, FUTURE. *Mol Interv*, 4:308-312.

35. Gold,M.H. and Goldman,M.P. (2004): 5-Aminolevulinic Acid Photodynamic Therapy: Where We Have Been and Where We Are Going. *Dermatol Surg*, 30:1077-1084.

36. Kufe,D.W., Pollock,R.E., Weichselbaum,R.R., Bast,R.C., Gansler,T.S., Holland,J.F., and Frei,E. (2003): *Holland-Frei Cancer Medicine*. Hamilton (ON): BC Decker,

37. Hamblin, M. R. Mechanisms of low level light therapy. http://www.photobiology.info/Hamblin.html. 2008. (GENERIC)
Ref Type: Electronic Citation

38. Uehlinger,P., Zellweger,M., Wagnieres,G., Juillerat-Jeanneret,L., van den,B.H., and Lange,N. (2000): 5-Aminolevulinic acid and its derivatives: physical chemical properties and protoporphyrin IX formation in cultured cells. *J Photochem Photobiol B.*, 54:72-80.

39. Juzeniene,A., Juzenas,P., Iani,V., and Moan,J. (2002): Topical application of 5-aminolevulinic acid and its methylester, hexylester and ocylester derivatives: considerations for dosimetry in mouse skin model. *Photochem Photoderm*, 76:329-334.

40. Palsson,S., Gustafsson,L., Bendsoe,N., Soto,T.M., ndersson-Engels,S., and Svanberg,K. (2003): Kinetics of the superficial perfusion and temperature in connection with photodynamic therapy of basal cell carcinomas using esterified and non-esterified 5-aminolaevulinic acid. *Br.J Dermatol.*, 148:1179-1188.

41. Juzeniene,A., Juzenas,P., Ma,L.-W., Iani,V., and Moan,J. (2006): Topical application of 5-aminolaevulinic acid, methyl 5-aminolaevulinate and hexyl 5-aminolaevulinate on normal human skin. *Br J Dermatol*, 155:791-799.

42. Kasche,A., Luderschmidt,S., Ring,J., and Hein,R. (2006): Photodynamic therapy induces less pain in patients treated with methyl aminolevulinate compared to aminolevulinic acid. *J Drugs Dermatol.*, 5:353-356.

43. Valentine,R.M., Ibbotson,S.H., Brown,C.T., Wood,K., and Moseley,H. (2011): A quantitative comparison of 5-aminolaevulinic Acid- and methyl aminolevulinate-induced fluorescence, photobleaching and pain during photodynamic therapy. *Photochem Photobiol.*, 87:242-249.

44. Rud,E., Gederaas,O., Hogset,A., and Berg,K. (2000): 5-aminolevulinic acid, but not 5-aminolevulinic acid esters, is transported into adenocarcinoma cells by system BETA transporters. *Photochem Photobiol.*, 71:640-647.

45. Wiegell,S.R., Stender,I.-M., Na,R., and Wulf,H.C. (2003): Pain Associated With Photodynamic Therapy Using 5-Aminolevulininc Acid or 5-Aminolevulinic Acid Methylester on Tape-Stripped Normal Skin. *Arch Dermatol*, 139:1173-1177.

46. Castano,A.P., Demidova,T.N., and Hamblin,M.R. (2004): Mechanisms in photodynamic therapy: part one- photosensitizers, photochemistry and cellular localization. *Photodiagnosis and Photodynamic Therapy*, 1:279-293.

47. Gederaas,O., Holroyd,A., and Brown,S. (2001): 5-Aminolevulinic acid methyl ester transport on amino acid carriers in a human colon adenocarcinoma cell line. *Photochem Photobiol*, 73:164-169.

48. Issa,M.C. and Manela-Azulay,M. (2010): Photodynamic therapy: a review of the literature and image documentation. *An.Bras.Dermatol.*, 85:501-511.

49. Henderson,B.W. and Dougherty,T.J. (1992): How does photodynamic therapy work? *Photochem Photobiol*, 55:145-157.

50. Gold,M.H., Rao,J., Goldman,M.P., Bridges,T.M., Bradshaw,V.L., Boring,M.M., and Guider,A.N. (2005): A multicenter clinical evaluation of the treatment of mild to moderate inflammatory acne vulgaris of the face with visible blue light in comparison to topical 1% clindamycin antibiotic solution. *J Drugs Dermatol.*, 4:64-70.

51. Hestdal, K. and Fekete, C. FDA Approves Metvixia/Aktilite Photodynamic Therapy. drugs.com . 2008. (GENERIC)
Ref Type: Electronic Citation

52. Castano,A.P., Mroz,P., and Hamblin,M.R. (2006): Photodynamic therapy and anti-tumour immunity. *Nat Rev Cancer.*, 6:535-545.

53. Heinzelmann-Schwarz,V., Fedier,A., Hornung,R., Walt,H., Haller,U., and Fink,D. (2003): Role of p53 and ATM in photodynamic therapy-induced apoptosis. *Lasers Surg Med.*, 33:182-189.

54. Oleinic,N.L., Morris,R.L., and Belichenko,I. (2002): The role of apoptosis in response to photodynamic therapy: what, where, why, and how. *Photochem Photobiol Sci.*, 1:1-21.

55. Lavie,G., Kaplinsky,C., Toren,A., Aizman,I., Meruelo,D., Mazur,Y., and Mandel,M. (1999): A photodynamic pathway to apoptosis and necrosis induced by dimethyl tetrahydroxyhelianthrone and hypericin in leukaemic cells: possible relevance to photodynamic therapy. *Br J Cancer.*, 79:423-432.

56. Luo,Y. and Kessel,D. (1997): Initiation of apoptosis versus necrosis by photodynamic therapy with chloroaluminum phthalocyanine. *Photochem Photobiol.*, 66:479-483.

57. Balkwill,F., Charles,K.A., and Mantovani,A. (2005): Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell.*, 7:211-217.

58. Canti,G., Lattuada,D., Nicolin,A., Taroni,P., Valentini,G., and Cubeddu,R. (1994): Antitumor immunity induced by photodynamic therapy with aluminum disulfonated phthalocyanines and laser light. *Anticancer Drugs.*, 5:443-447.

59. Bhowmick,R. and Girotti,A.W. (2009): Signaling events in apoptotic photokilling of 5-aminolevulinic acid-treated tumor cells: inhibitory effects of nitric oxide. *Free Radic.Biol Med.*, 47:731-740.

60. Wang,I., ndersson-Engels,S., Nilsson,G.E., Wardell,K., and Svanberg,K. (1997): Superficial blood flow following photodynamic therapy of malignant non-melanoma skin tumours measured by laser Doppler perfusion imaging. *Br.J Dermatol.*, 136:184-189.

61. Reeves,K.J., Reed,M.W.R., and Brown,N.J. (2009): Is nitric oxide important in photodynamic therapy? *J Photochem Photobiol B.*, 95:141-147.

62. Reeves,K.J., Reed,M.W.R., and Brown,N.J. (2010): The role of nitric oxide in the treatment of tumors with aminolaevulinic acid-induced photodynamic therapy. *J Photochem Photobiol B.*, 101:224-232.

63. Pogue,B.W., O'Hara,J.A., Goodwin,I.A., Wilmot,C.J., Fournier,G.P., Akay,A.R., and Swartz,H. (2002): Tumor PO(2) changes during photodynamic therapy depend upon photosensitizer type and time after injection. *Comp Biochem Physiol A Mol Integr Physiol*, 132:177-184.

64. Arits,A.H., van de Weert,M.M., Nelemans,P.J., and Kelleners-Smeets,N.W. (2010): Pain during topical photodynamic therapy: uncomfortable and unpredictable. *J Eur Acad.Dermatol Venereol.*, [Epub ahead of print]:

65. Stender,I.-M., Borgbjerg,F.M., Villumsen,J., Lock-Andersen,J., and Wulf,H.C. (2006): Pain induced by photodynamic therapy of warts. *Photodermatol Photoimmunol Photomed*, 22:304-309.

66. Warren,C.B., Karai,L.J., Vidimos,A., and Maytin,E.V. (2009): Pain associated with aminolevulininc acid-photodynamic therapy of skin disease. *J Am.Acad.Dermatol.*, 61:1033-1043.

67. Wennberg,A.M. (2005): Pain, pain relief and other practical issues in photodynamic therapy. *Australas J Derm*, 46:S3-S4

68. Wiegell,S.R., Skiveren,J., Philipsen,P.A., and Wulf,H.C. (2008): Pain during photodynamic therapy is associated with protoporphyrin IX fluorescence and fluence rate. *Br.J.Dermatol*, 158:727-733.

69. Grapengiesser,S., Ericson,M., Gudmundsson,F., Larko,O., Rosen,A., and Wennberg,A.M. (2002): Pain caused by photodynamic therapy of skin cancer. *Clin.Exp.Dermatol*, 27:493-497.

70. Algermissen,B., Osterloh,D., Philipp,C.M., and Berlien,H.-P. (2003): Management of ALA-PDT induced pain sensations. *Med Laser Appl*, 18:57-64.

71. Steinbauer,J., Schreml,S., Karrer,S., Ackermann,G., Babilas,P., Landthaler,M., and Szeimies,R.M. (2009): Phototoxic reactions in healthy volunteers following photodynamic therapy with methylaminolevulinate cream or with cream containing 5-aminolevulinic acid: a phase II, randomized study. *Photodermatol Photoimmunol Photomed*, 25:270-275.

72. Muller,W.E. and Snyder,S.H. (1977): delta-Aminolevulinic acid: influences on synaptic GABA receptor binding may explain CNS symptoms of porphyria. *Ann.Neurol.*, 2:340-342.

73. Radakovic-Fijan,S., Blecha-Thalhammer,U., Schleyer,V., Szeimies,R.M., Zwingers,T., Honigsmann,H., and Tanew,A. (2005): Topical aminolaevulinic acid-based photodynamic therapy as a treatment option for psoriasis? Results of a randomized, observer-blinded study. *Br.J.Dermatol*, 152:279-283.

74. von Oosten,E.J., Kijupers,D.I.M., and Thissen,M.R.T.M. (2006): Different pain sensations in photodynamic therapy of nodular basal cell carcinoma. Results from a prospective trial and a review of the literature. *Photodiagnosis and Photodynaic Therapy*, 3:61-68.

75. Kleinpenning,M.M., Wolberink,E.W., Smits,T., Blokx,W.A., van de Kerkhof,P.C., van Erp,P.E., and Gerritsen,R.M. (2010): Fluorescence diagnosis in actinic keratosis and squamous cell carcinoma. *Photodermatol Photoimmunol Photomed*, 26:297-302.

76. Warloe,T., Peng,Q., Moan,J., and et al. (1992): Photochemotherapy of multiple basal cell carcinoma with endogenous porphyrins induced by topical application of 5-aminolevulinic acid. In: *Photodynamic therapy and biomedical lasers*, edited by P.Spinelli, et al, pp. 449-453. Elsevier Science Publishers BV, Amsterdam.

77. Gerweck,L.E. (1985): Hyperthermia in cancer therapy: the biological basis and unresolved questions. *Cancer Res.*, 45:3408-3414.

78. Cottrell,W.J., Paquette,A.D., Keymel,K.R., Foster,T.H., and Oseroff,A.R. (2008): Irradiance-dependent photobleaching and pain in delta-aminolevulinic acid-photodynamic therapy of superficial basal cell carcinomas. *Clin Cancer Res.*, 14:4475-4483.

79. Ericson,M., Sandberg,C., Stenquist,B., Gudmundson,F., Karlsson,M., Ros,A.-M., Rosen,A., Larko,O., Wennberg,A.M., and Rosdahl,I. (2004): Photodynamic therapy of actinic keratosis at varying fluence rates: assessment of photobleaching, pain and primary clinical outcome. *Br.J Dermatol.*, 151:1204-1212.

80. Middelburg,T.A., Van,Z.F., de Bruijn,H.S., Van Der Ploeg-van den Heuvel, Sterenborg,H.J., Neumann,H.A., De Haas,E.R., and Robinson,D.J. (2010): Fractionated illumination at low fluence rate photodynamic therapy in mice. *Photochem Photobiol.*, 86:1140-1146.

81. Sterenborg,H.J., de Wolf,J., Koning,M., Kruijt,B., van den Heuvel,A., and Robinson,D.J. (2004): Phosphorescence-Fluorescence ratio imaging for monitoring the oxygen status during photodynamic therapy. *Opt Express*, 12:1873-1878.

82. de Bruijn,H.S., Kruijt,B., Van Der Ploeg-van den Heuvel, Sterenborg,H.J., and Robinson,D.J. (2007): Increase in protoporphyrin IX after 5-aminolevulinic acid based photodynamic therapy is due to local re-synthesis. *Photochem Photobiol Sci*, 6:857-864.

83. De Haas,E.R., Kruijt,B., Sterenborg,H.J., Neumann,H.A., and Robinson,D.J. (2006): Fractionated Illumination Significantly Improves the Response of Superficial Basal Cell Carcinoma to Aminolevulinic Acid Photodynamic Therapy. *J Invest Dermatol*, 126:2679-2686.

84. van der Veen,N., Hebeda,K.M., de Bruijn,H.S., and Star,W.M. (1999): Photodynamic effectiveness and vasoconstriction in hairless mouse skin after topical 5-aminolevulinic acid and single- or two-fold illumination. *Photochem Photobiol.*, 70:921-929.

85. Oberdanner,C.B., Plaetzer,K., Kiesslich,T., and Krammer,B. (2005): Photodynamic treatment with fractionated light decreases production of reactive oxygen species and cytotoxicity in vitro via regeneration of glutathione. *Photochem Photobiol*, 81:609-613.

86. Lindeburg,K., Brogaard,H., and Jemec,G. (2007): Pain and Photodynamic Therapy. *Dermatology*, 215:206-208.

87. Peng,Q., Juzeniene,A., Chen,J., Svaasand,L.O., Warloe,T., Giercksky,K.E., and Moan,J. (2008): Lasers in medicine. *Rep Prog Phys*, 71:1-28.

88. Fritsch,C., Stege,H., Saalmann,G., Goerz,G., Ruzicka,T., and Krutmann,J. (1997): Green light is effective and less painful than red light in photodynamic therapy of facial solar keratoses. *Photodermatol Photoimmunol Photomed*, 13:181-185.

89. Fink-Puches,R., Hofer,A., Smolle,J., Kerl,H., and Wolf,P. (1997): Primary clinical response and long-term follow-up of solar keratoses treated with topically applied 5-aminolevulinic acid and irradiation by different wave bands of light. *J Photochem Photobiol B.*, 41:145-151.

90. Morton,C.A., Whitehurst,C., Moore,J.V., and MacKie,R.M. (2000): Comparison of red and green light in the treatment of Bowen's disease by photodynamic therapy. *Br.J Dermatol.*, 143:767-772.

91. Palm,M.D. and Goldman,M.P. (2011): Safety and efficacy comparison of blue versus red light sources for photodynamic therapy using methyl aminolevulinate in photodamaged skin. *J Drugs Dermatol.*, 10:53-60.

92. Ma,L.W., Nielsen,K.P., Iani,V., and Moan,J. (2007): A new method for photodynamic therapy of melanotic melanoma -- effects of depigmentation with violet light photodynamic therapy. *J Environ.Pathol.Toxicol.Oncol.*, 26:165-172.

93. Gholam,P., Denk,K., Sehr,T., Enk,A., and Hartmann,M. (2010): Factors influencing pain intensity during topical photodynamic therapy of complete cosmetic units for actinic keratoses. *J Am.Acad.Dermatol.*, 63:213-218.

94. Corti,M.A. and Mainetti,C. (2010): Methylaminolevulinic acid-based photodynamic therapy: the patient's view. *Photomed Laser Surg.*, 28:697-702.

95. Holmes,M.V., Dawe,R.S., Ferguson,J., and Ibbotson,S.H. (2003): A randomized, double-blind, placebo-controlled study of the efficacy of tetracaine gel (AmetopR) for pain relief during topical photodynamic therapy. *Br.J Dermatol.*, 150:337-340.

96. Miller,I., Nielsen,J., Lophaven,S., and Jemec,G. (2011): Factors related to pain during routine photodynamic therapy: a descriptive study of 301 patients. *J Eur Acad.Dermatol Venereol.*, 10-3083.

97. Dole,K.C., Chen,Q., Hetzel,F.W., Whalen,L.R., Blanc,D., and Huang,Z. (2005): Effects of Photodynamic Therapy on Peripheral Nerve: *In Situ* Compound-Action Potentials Study in a Canine Model. *Photomed Laser Surg.*, 23:172-176.

98. Sandberg,C., Stenquist,B., Rosdahl,I., Ros,A.-M., Synnerstad,I., Karlsson,M., Gudmundson,F., Ericson,M., Larko,O., and Wennberg,A.M. (2006): Important factors for pain during photodynamic therapy for actinic keratosis. *Acta Derm Venereol*, 86:404-408.

99. Brooke,R.C., Sinha,A., Sidhu,M.K., Watson,R.E., Church,M.K., Friedmann,P.S., Clough,G.F., and Rhodes,L.E. (2006): Histamine is released following aminolevulinic acid-photodynamic therapy of human skin and mediates an aminolevulinic acid dose-related immediate inflammatory response. *J Invest Dermatol.*, 126:2296-2301.

100. Caterina,M.J., Schumacher,M.A., Tominaga,M., Rosen,T.A., Levine,J.D., and Julius,D. (1997): The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature.*, 389:816-824.

101. Halldin,C.B., Paoli,J., Sandberg,C., Ericson,M.B., and Wennberg,A.M. (2008): Transcutaneous electrical nerve stimulation for pain relief during photodynamic therapy of actinic keratoses. *Acta Derm Venereol.*, 88:311-313.

102. Leff,D.R., Nortley,M., Dang,V., and Bhutiani,R.P. (2007): The effect of local cooling on pain perception during infiltration of local anaesthetic agents, a prospective randomised controlled trial. *Anaesthesia.*, 62:677-682.

103. Proudfoot,C.J., Garry,E.M., Cottrell,D.F., Rosie,R., Anderson,H., Robertson,D.C., Fleetwood-Walker,S.M., and Mitchell,R. (2006): Analgesia mediated by the TRPM8 cold receptor in chronic neuropathic pain. *Curr.Biol.*, 16:1591-1605.

104. Tyrrell,J., Campbell,S.M., and Curnow,A. (2010): The effect of air cooling pain relief on protoporphyrin IX photobleaching and clinical efficacy during dermatological photodynamic therapy. *J Photochem Photobiol B.*,

105. Halldin,C.B., Paoli,J., Sandberg,C., Gonzalez,H., and Wennberg,A.M. (2009): Nerve blocks enable adequate pain relief during topical photodynamic therapy of field cancerization on the forehead and scalp. *Br.J Dermatol.*, 160:795-800.

106. Paoli,J., Halldin,C., Ericson,M.B., and Wennberg,A.M. (2008): Nerve blocks provide effective pain relief during topical photodynamic therapy for extensive facial actinic keratoses. *Clin Exp Dermatol.*, 33:559-564.

107. Serra-Guillen,C., Hueso,L., Nagore,E., Vila,M., Llombart,B., Requena,C.C., Botella-Estrada,R., Sanmartin,O., faro-Rubio,A., and Guillen,C. (2009): Comparative study between cold air analgesia and supraorbital and supratrochlear nerve block for the management of pain during photodynamic therapy for actinic keratoses of the frontotemporal zone. *Br.J Dermatol.*, 161:353-356.

108. Soriano,T.T., Lask,G.P., and Dinehart,S.M. (2005): Anaesthesia and analgesia. In: *Surgery of the Skin: Procedural Dermatology*, edited by J.K.Robinson, et al, pp. 39-58. MO:Elsevier Mosby, St Louis.

109. Berking,C., Herzinger,T., Flaig,M.J., Brenner,M., Borelli,C., and Degitz,K. (2007): The efficacy of photodynamic therapy in actinic cheilitis of the lower lip: a prospective study of 15 patients. *Dermatol Surg.*, 33:825-830.

110. Borelli,C., Herzinger,T., Merk,K., Berking,C., Kunte,C., Plewig,G., and Degitz,K. (2007): Effect of subcutaneous infiltration anesthesia on pain in photodynamic therapy: a controlled open pilot trial. *Dermatol Surg.*, 33:314-318.

111. Tan,G., Fukui,T., Jensen,M.P., Thornby,J., and Waldman,K.L. (2010): Hypnosis treatment for chronic low back pain. *Int.J Clin Exp Hypn.*, 58:53-68.

112. Langan,S.M. and Collins,P. (2006): Randomized, double-blind, placebo-controlled prospective study of the efficacy of topical anaesthesia with a eutetic mixture of

lignocaine 2.5% and prilocaine 2.5% for topical 5-animolevulinic acid-photodynamic therapy for extensive scalp keratoses. *Br.J Dermatol.*, 154:146-149.

113. Touma,D., Yaar,M., Whitehead,S., Konnikov,N., and Gilchrest,B.A. (2004): A trial of short incubation, broad-area photodynamic therapy for facial actinic keratoses and diffuse photodamage. *Arch.Dermatol.*, 140:33-40.

114. Skiveren,J., Haedersdal,M., Philipsen,P.A., Wiegell,S.R., and Wulf,H.C. (2006): Morphine gel 0.3% does not relieve pain during topical photodynamic therapy: a randomized, double-blind, placebo-controlled study. *Acta Derm Venereol*, 86:409-411.

115. Pagliaro,J., Elliott,T., Bulsara,M., King,C., and Vinciullo,C. (2004): Cold air analgesia in photodynamic therapy of basal cell carcinomas and Bowen's disease: an effective addition to treatment: a pilot study. *Dermatol Surg.*, 30:63-66.

116. Noguchi,P., Wallace,R., Johnson,J., Earley,E.M., O'Brien,S., Ferrone,S., Pellegrino,M.A., Milstein,J., Needy,C., Browne,W., and Petricciani,J. (1979): Characterization of WiDr: a Human Colon Carcinoma Cell Line. *In Vitro*, 15:401-408.

117. Rodrigues,N.R., Rowan,A., Smith,M.E., Kerr,I.B., Bodmer,W.F., Gannon,J.V., and Lane,D.P. (1990): p53 mutations in colorectal cancer. *Proc.Natl.Acad.Sci.U.S.A.*, 87:7555-7559.

118. Noodt,B.B., Berg,K., Stokke,T., Peng,Q., and Nesland,J.M. (1996): Apoptosis and necrosis induced with light and 5-aminolaevulinic acid-derived protoporphyrin IX. *Br.J.Cancer*, 74:22-29.

119. Giard,D.J., Aaronson,S.A., Todaro,G.J., Arnstein,P., Kersey,J.H., Dosik,H., and Parks,W.P. (1973): In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J.Natl.Cancer Inst.*, 51:1417-1423.

120. Choi,K.H., Hama-Inaba,H., Wang,B., Haginoya,K., Odaka,T., Yamada,T., Hayata,I., and Ohyama,H. (2000): UVC-induced apoptosis in human epithelial tumor A431 cells: sequence of apoptotic changes and involvement of caspase (-8 and -3) cascade. *J Radiat.Res.(Tokyo).*, 41:243-258.

121. Huang,W. and Vidimos,A. (2000): Topical anesthetics in dermatology. *J Am.Acad.Dermatol.*, 43:286-298.

122. Das, K. C. and Misra, H. P. Lidocaine: a hydroxyl radical scavenger and singlet oxygen quencher. Mol Cell Biochem 115, 179-185. 1992. (GENERIC)
Ref Type: Journal (Full)

123. Juzenas,P., Juzeniene,A., Iani,V., and Moan,J. (2009): Depth profile of protoporphyrin IX fluorescence in an amelanotic mouse melanoma model. *Photochem Photobiol*, 85:760-764.

124. Wang,X., Wang,H., and Huang,Z. (2010): Cutaneous Photosensitization Induced by Exposure to Colour Cathode Ray Tube Monitor Following Topical 5-Aminolaevulinic Acid-based Photodynamic Therapy: A Case Report . *Acta Derm Venereol*, 90:545-547.

125. Moan,J., Iani,V., and Ma,L.-W. (1996): Choice of the proper wavelength for photochemotherapy. In: "Photochemotherapy: Photodynamic Therapy and Other Modalites", Benjamin Ehrenberg, Giulio Jori, Johan Moan, Editors. *Proc SPIE*, 2625:544-549.

126. Gerritsen,M.J., Smits,T., Kleinpenning,M.M., van de Kerkhof,P.C., and van Erp,P.E. (2009): Pretreatment to enhance protoporphyrin IX accumulation in photodynamic therapy. *Dermatology*, 218:193-202.

127. Brown,M.B., Traynor,M.J., Martin,G.P., and Akomeah,F.K. (2008): Transdermal drug delivery systems: skin perturbation devices. *Methods Mol Biol*, 437:119-139.

128. Drakuliæ,B.J., Juraniæ,I.O., Eriæ,S., and Zloh,M. (2008): Role of complexes formation between drugs and penetration enhancers in transdermal delivery. *Int J Pharm*, 363:40-49.

129. Saha,S.A., Kordouni,M.R., Siddiqui,M., and Arora,R.R. (2006): Methemoglobinemia-induced cardio-respiratory failure secondary to topical anesthesia. *Am J Ther*, 13:545-549.

130. Egekvist,H., Bjerring,P., and Arendt-Nielsen,L. (1999): Pain and mechanical injury of human skin following needle insertions. *Eur J Pain*, 3:41-49.

131. Donnelly,R.F., Morrow,D.I., McCarron,P.A., Woolfson,A.D., Morrissey,A., Juzenas,P., Juzeniene,A., Iani,V., McCarthy,H.O., and Moan,J. (2008): Microneedle-mediated intradermal delivery of 5-aminolevulinic acid: potential for enhanced topical photodynamic therapy. *J Control Release*, 129:154-162.

132. Bond,J.R. and Barry,B.W. (1988): Hairless mouse skin is limited as a model for assessing the effects of penetration enhancers in human skin. *J Invest Dermatol*, 90:810-813.

133. Attili,S.K., Lesar,A., McNeill,A., Camacho-Lopez,M.A., Moseley,H., Ibbotson,S.H., Samuel,I.D., and Ferguson,J. (2009): An open pilot study of ambulatory photodynamic therapy using a wearable low-irradiance organic light-emitting diode light source in the treatment of nonmelanoma skin cancer. *Br J Dermatol*, 161:170-173.

134. Moseley,H., Allen,J.W., Ibbotson,S.H., Lesar,A., McNeill,A., Camacho-Lopez,M.A., Samuel,I.D., Sibbett,W., and Ferguson,J. (2006): Ambulatory photodynamic therapy: a new concept in delivering photodynamic therapy. *Br J Dermatol*, 154:747-750.

135. Clark, C., Dawe, R. S., Moseley, H., Ferguson, J., and Ibbotson, S. H. The characteristics of erythema induced by topical 5-aminolevulinic acid photodynamic therapy. Photodermatol Photoimmunol Photomed 20, 105-107. 2004. (GENERIC)
Ref Type: Journal (Full)

136. Lesar,A., Ferguson,J., and Moseley,H. (2011): An investigation of the fluorescence induced by topical application of 5-aminolevulinic acid and methyl aminolevulinate at different body sites on normal human skin. *Photodiagnosis and Photodynaic Therapy*,

137. Fahey,J.W. and Talalay,P. (1999): Antioxidant functions of sulforaphane: a potent inducer of Phase II detoxyfication enzymes. *Food Chem Toxicol*, 37:973-979.

138. Fahey,J.W., Zhang,Y., and Talalay,P. (1997): Broccoli sprouts: an exceptionally rich source of enzymes that protect against chemical carcinogenes. *Proc.Natl.Acad.Sci.U.S.A.*, 94:10367-10372.

139. Zhang,Y., Talalay,P., Cho,C.G., and Prosner,G.H. (1992): A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc.Natl.Acad.Sci.U.S.A.*, 89:2399-2403.

140. Zhang,Y. and Talalay,P. (1994): Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. *Cancer Res.*, 54:1976s-1981s.

141. Glover,R.A., Bailey,C.S., Barret,K.E., Wasserman,S.I., and Gigli,I. (1990): Histamine release from rodent and human mast cells induced by protoporphyrin and ultraviolet light: studies of the mechanism of mast-cell activation in erythropoetic protoporphyria. *Br J Dermatol*, 122:501-512.

142. Berg,K. and Moan,J. (1997): Lysosomes and microtubules as targets for photochemotherapy of cancer. *Photochem Photobiol*, 65:403-409.

143. Tromberg,B.J., Orenstein,A., Kimel,S., Barker,S.J., Hyatt,J., Nelson,J.S., and Berns,M.W. (1990): In vivo tumor oxygen tension measurements for the evaluation of the efficiency of photodynamic therapy. *Photochem Photobiol*, 52:375-385.

144. Kaminer,M.S., Murphy,G.F., Zweiman,B., and Lavker,R.M. (1995): Connective Tissue Mast Cells Exhibit Time-Dependent Degranulation Heterogeneity . *Clin Diagn Lab Immunol*, 2:297-301.

1

2

3

4

5